AUTOREGULATION OF PRO-INFLAMMATORY PROTEIN EXPRESSION IN MURINE MACROPHAGES BY NITRIC OXIDE

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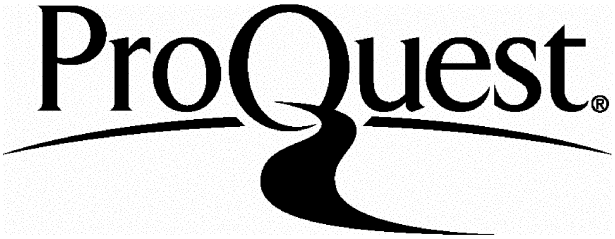

ABSTRACT

Macrophages are effector cells of the immune system that participate in host defence through the killing and removal of bacteria, tumour cells and other pathogenic stimuli. One mechanism of cytotoxicity and cytostasis employed by the activated macrophage is the up-regulation/expression of the enzyme inducible nitric oxide synthase (iNOS) and subsequent production of nitric oxide (NO).

A key transcription factor activated in response to pathogenic stimuli, including bacterial lipopolysaccharide (LPS), is nuclear factor-κB (NF-κB); this factor is responsible for the up-regulation of numerous pro-inflammatory proteins such as iNOS, cyclo-oxygenase–2 (COX–2) and interleukin-6 (IL-6). The studies described in this thesis investigated the hypothesis that NO can modulate the activation profile of the macrophage, and pro-inflammatory protein expression, via regulation of NF-κB activity. Such a mechanism may represent a feedback pathway by which NO might regulate its own production through effects on iNOS expression. Potential mechanisms underlying regulation of NF-κB activity and pro-inflammatory protein expression by NO were investigated using cells from NOS knockout mice and activators and inhibitors of soluble guanylate cyclase.

Herein it is demonstrated that endogenous NO has a biphasic effect on NF-κB activation by which NO can both up- and down-regulate pro-inflammatory protein expression. At low concentrations of NO both LPS-stimulated NF-κB activity and pro-inflammatory protein expression were augmented while at high concentrations NO exerted an inhibitory effect. Thus, NO acts in both a pro- and anti-inflammatory manner depending on the local concentration, initially augmenting macrophage activation and then assisting in the down-regulation of the response. These opposing actions of NO may involve the activity of constitutive and inducible isoforms of NOS in addition to cGMP –dependent and –independent pathways.

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PUBLICATIONS

Work described in this thesis has given rise to the following publications:

Connelly, L., Palacios-Callander, M., Moncada, S., and Hobbs, A. J. (2000) Endogenous nitric oxide modulates murine macrophage activation by lipopolysaccharide. *Nitric Oxide* **4:** 297

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1400W	[N-(3-aminomethyl)benzylacetamine, 2HCl]
ADP	adenosine diphosphate
AG	aminoguanidine
A-kinase	cAMP-dependent protein kinase
ANP	atrial natriuretic peptide
AP-1	activating protein 1
BAEC	bovine aortic endothelial cells
BH_4	tetrahydrobiopterin
BSO	buthionine sulfoximine
cAMP	adenosine-3'5'-monophosphate
CAT	chloramphenicol acetyltransferase
cGMP	guanosine-3'5'-monophosphate
COX-1	cyclo-oxygenase 1
COX-2	cyclo-oxygenase 2
CRE	cAMP-response element
CREB	cAMP-response element binding protein
DEA-NO	diethylamine NONOate
DMEM	Dulbecco's modified eagles medium
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDRF	endothelium derived relaxing factor
EDTA	ethylenediaminetetracetic acid
EGFα	epidermal growth factor alpha
EGTA	ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetracetic acid
EMSA	electrophoretic mobility shift assay
eNOS	endothelial nitric oxide synthase
EP	E-prostanoid
FAD	flavin adenine dinucleotide
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate conjugated
FMN	flavin mononucleotide
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

GAS	gamma activated site
G-kinase	cGMP-dependent protein kinase
GM-CSF	granulocyte macrophage colony stimulating factor
gp80	IL-6 receptor
GSH	reduced glutathione
GSNO	S-nitrosoglutathione
GTN	glycerol trinitrate
GTP	guanosine 5'-triphosphate
H_2O_2	hydrogen peroxide
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HRP	horse radish peroxidase
HUVEC	human umbilical vein endothelial cell
ICAM-1	intracellular adhesion molecule 1
IFNβ	interferon beta
IFNγ	interferon gamma
IKK	IκB kinase
IL-1	interleukin 1
IL-1R	interleukin 1 receptor
IL-4	interleukin 4
IL-6	interleukin 6
IL-8	interleukin 8
IL-10	interleukin 10
IL-12	interleukin 12
iNOS	inducible nitric oxide synthase
IRAK	IL-1 receptor associated kinase
IRF-1	interferon regulatory factor 1
IκB	inhibitor of κB
JAK	janus kinase
JNK	c-jun N-terminal kinase
KO	knockout
LBP	LPS binding protein
L-NAME	N^G-nitro-L-arginine methyl ester
L-NMA	N^G-methyl-L-arginine

LPS	lipopolysaccharide
LT	leukotriene
LTP	long term potentiation
Mal	MyD88-adaptor-like
M-CSF	macrophage colony stimulating factor
MAP3K	mitogen activated protein kinase kinase kinase
MAPK	mitogen activated protein kinase
NAC	N-acetyl cysteine
NADPH	nicotinamide adenine dinucleotide phosphate
NEMO	NF-kappaB essential modulator
NF-IL6	nuclear factor interleukin 6
NF-κB	nuclear factor kappa B
NIK	NF-κB inducing kinase
NLS	nuclear localisation sequence
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO^+	nitrosonium ion
NO_2^-	nitrite
NO_3^-	nitrate
NOHA	N^G-hydroxy-L-arginine
NOS	nitric oxide synthase
O_2^-	superoxide
ODQ	1H-(1,2,4)oxadiazol(4,3-a)quinoxalin-1-one
$OH^\cdot$	hydroxyl radical
$ONOO^-$	peroxynitrite
PBS	phosphate buffered saline
PDE	cyclic nucleotide phosphodiesterase
PDTC	pyrrolidine dithiocarbamate
PDZ	PSD-95, Dlg, ZO-1 homology
PG	prostaglandin
PI-3K	phosphatidylinositol-3-OH-kinase
PKC	protein kinase C

PMA	phorbol myristate
PSD-95	postsynaptic density-95
ROI	reactive oxygen intermediate
RT-PCR	reverse transcriptase polymerase chain reaction
S. typhosa	*Salmonella typhosa*
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
sGC	soluble guanylate cyclase
SH2	src homology 2
SIN-1	3-morpholinosydnonimine
SNAC	S-nitroso-N-acetylcysteine
SNAP	S-nitroso-penicillamine
SNP	sodium nitroprusside
SOD	superoxide dismutase
STAT	signal transducer and activator of transcription
TAK-1	TGFβ-activated kinase
TGFβ	transforming growth factor beta
TLR	toll-like receptor
TNF-RE	tumour necrosis factor response element
TNFα	tumour necrosis factor alpha
TNFβ	tumour necrosis factor beta
TRAF-6	tumour necrosis factor associated factor 6
TRIKA2	TRAF-6 regulated IKK activator 2
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
WT	wild type
γ-IRE	gamma interferon response element

CHAPTER ONE
INTRODUCTION

1. INTRODUCTION

1.1 OVERVIEW

Nitric oxide (NO) has been shown to play a role in a wide range of physiological and pathophysiological processes (Moncada *et al.*, 1991; Hobbs *et al.*, 1999). The discovery of an endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) led to the identification of NO as the major endogenous vasodilator in mammals (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Furchgott, 1988). Subsequently, NO has also been found to be synthesised and released by neurons and demonstrated to be involved in neurotransmission in the central and peripheral nervous system (Garthwaite *et al.*, 1988; Hobbs & Gibson, 1990). In addition to its signalling role in the vasculature and nervous system, NO is also released as an effector molecule by cells of the immune system, mediating cytostatis and cytotoxicity (Hibbs *et al.*, 1988).

While in many physiological processes NO is an essential mediator, aberrant production of NO is associated with a number of pathologies. For example, excessive production of NO is linked to the profound vasodilation observed during sepsis, which leads to organ failure (Wright *et al.*, 1992; Titheradge, 1999). The unregulated production of NO has also been associated with the excitotoxicity and neuronal damage observed during stroke and the neurodegeneration in Parkinson's and Huntington's Diseases (Christopherson & Bredt, 1997; Dawson & Dawson, 1998). Conversely, decreased production of NO in the vasculature is associated with atherosclerosis and hypertension (Moncada *et al.*, 1991). Therefore, an increased understanding as to how NO signalling is modulated during both physiological and pathological situations may aid the design of future therapies for these conditions.

1.2 NITRIC OXIDE SYNTHASE

1.2.1 Nitric oxide synthase properties

NO is synthesised via the five electron oxidation of a guanidino nitrogen on the amino acid L-arginine and is produced in equimolar quantities with its co-product, L-

citrulline (Bush *et al.*, 1992). The initial stage of the reaction involves the N-hydroxylation of L-arginine to form N^G-hydroxy-L-arginine (NOHA), the only identifiable intermediate (Stuehr *et al.*, 1991a). NOHA is then oxygenated to produce L-citrulline and NO.

The enzyme responsible for this reaction is nitric oxide synthase (NOS). This enzyme is enantiomer-specific in that it will metabolise L-arginine, but not D-arginine. In order to produce NO the enzyme requires the presence of the co-substrates; nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen and the co-factors: haem, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4) and calmodulin (reviewed by Stuehr, 1997). The monomeric NOS is a bidomain protein made up of a reductase and oxygenase domain joined by a calmodulin binding site. NOS is only active as a dimer of this subunit with dimerisation occuring via the binding of two oxygenase domains (Stuehr, 1997).

The C-terminal or reductase domain contains the binding sites for FAD, FMN and NADPH. The structure and function of the reductase domain shares homology with the protein cytochrome P450 reductase (Bredt *et al.*, 1991). The NADPH-binding site is at the extreme C-terminal and is followed by the binding sites for FAD and FMN (see Figure 1). The oxygenase domain is the site of binding for the haem which is coordinated by a conserved cysteine residue (Stuehr, 1997). BH_4 and L-arginine bind close to the haem site. While the reductase domain is predicted to have a modular structure with the consensus binding sites arranged in a linear sequence, the oxygenase domain is made up of a series of folds and the elements which form the binding sites are spread throughout the domain (Stuehr, 1999).

The flavins accept electrons from NADPH and shuttle them to the oxygenase domain. The reductase domain and oxygenase domain are joined by a calmodulin-binding site, which gates the flow of electrons between the flavins and haem group (Abu-Soud & Stuehr, 1993). At resting levels of Ca^{2+} (<100nM), constitutive NOS isoforms do not bind calmodulin and are inactive. Cellular activation leading to a rapid transient Ca^{2+} influx permits calmodulin binding, allowing the electrons derived from NADPH to be transferred to the haem group, a process which cannot occur when calmodulin is absent (Abu-Soud & Stuehr, 1993). By this mechanism NO production from a

constitutive NOS isoform can be rapidly switched on and off. Transfer of electrons occurs between the reductase and oxygenase domains of opposite subunits in the dimeric enzyme (Siddhanta *et al.*, 1998; Panda *et al.*, 2001; Sagami *et al.*, 2001), perhaps explaining why dimer formation is essential for enzyme activity.

L-arginine binds to the enzyme such that a guanidino nitrogen is positioned above the haem group (Crane *et al.*, 1998). Once the co-factors are reduced, electrons are shuttled along the enzyme to the haem group which binds and activates molecular oxygen and catalyses the N-hydroxylation of L-arginine. This reaction requires 1 mole of NADPH and 0.5 moles O_2. The haem is also the site of the second step in the reaction, the oxygenation of NOHA to form NO and L-citrulline, which requires 0.5 moles NADPH and 0.5 moles O_2 (Kwon *et al.*, 1990; Stuehr *et al.*, 1991a).

BH_4 binds close to the haem edge with a hydrogen bond forming between the N_3 of the pterin ring and the haem propionate (Raman *et al.*, 1998). It appears that BH_4 acts as a source of the second electron which is required for the hydroxylation of L-arginine. BH_4 is thus oxidised and a BH_3 free radical is formed (Hurshman *et al.*, 1999; Wei *et al.*, 2001). Mechanisms responsible for the recycling of BH_4 are unclear.

There are three isoforms of NOS, which share 50-60% sequence homology and are classified according to regulation of enzyme activation and cellular location. There are two constitutive NOS isoforms: endothelial NOS (eNOS) and neuronal NOS (nNOS), which are regulated via transient increases in intracellular free calcium. The third isoform is inducible NOS (iNOS) and its activity is independent of calcium and controlled via regulation of enzyme expression (Forstermann & Kleinert, 1995). The structure of each isoform monomer is shown in Figure 1.

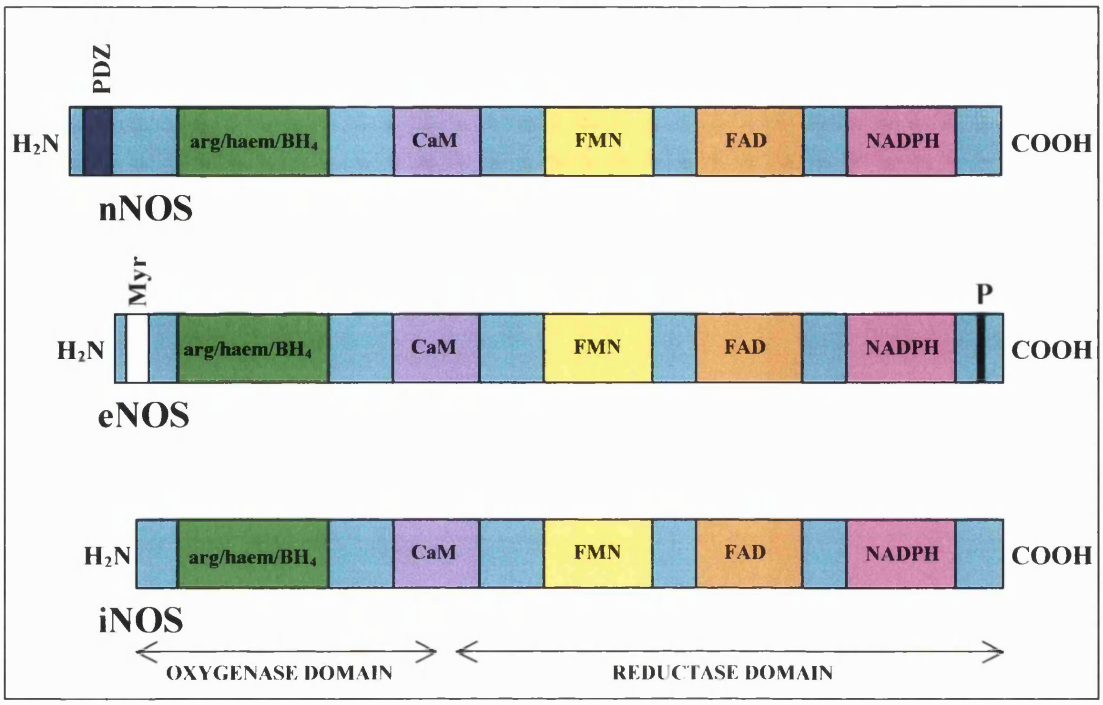


FIGURE 1. Structure of NOS isozymes showing substrate and co-factor binding sites. PDZ - postsynaptic density 95 (PSD-95), Dlg, ZO-1 homology domain; arg – L-arginine; BH_4-tetrahydrobiopterin; CaM – calmodulin; FMN – flavin mononucleotide; FAD – flavin adenine dinucleotide; NADPH – nitotinamide adenine diphosphate; P – phosphorylation site; Myr – myristoylation site; nNOS – neuronal nitric oxide synthase; eNOS – endothelial nitric oxide synthase; iNOS – inducible nitric oxide synthase

1.2.2 Neuronal nitric oxide synthase

Neuronal NOS was first purified from rat and porcine cerebellum (Bredt & Snyder, 1990; Mayer *et al.,* 1990) and although the majority of nNOS is expressed in the central and peripheral nervous system it has also been found in human skeletal muscle (Nakane *et al.*, 1993). It is a 150-160kDa protein that is mainly soluble and requires Ca^{2+}/calmodulin for activity.

The nNOS isoform differs from eNOS and iNOS in that it has an additional 200 amino acids at the N-terminal which form a structural motif known as a postsynaptic density 95 (PSD-95), Dlg, ZO-1 homology (PDZ) domain. The PDZ domain recognises and binds to PDZ domains in other proteins and thus mediates nNOS associations. For example, nNOS associates with the α1-synotrophin in the sarcolemma of skeletal muscle and with PSD-95 in neurons (Brenman *et al.*, 1996). In the brain, nNOS binds to PSD-95 via PDZ domains and through this interaction becomes associated with the N-methyl-D-aspartate (NMDA) receptor (Brenman *et al.*, 1996; Christopherson *et al.*, 1999). The activation of the NMDA receptor by

glutamate leads to the receptor channel opening and the influx of Ca^{2+} which initiates activation of nNOS (Garthwaite, 1991), an essential component of glutamergic transmission involved in long term potentiation (LTP).

In the peripheral nervous system NO produced by nNOS acts as a non-adrenergic, non-cholinergic neurotransmitter which mediates the actions of autonomic motor neurons on vascular and non-vascular smooth muscle (Moncada *et al.*, 1991). For example, this has been demonstrated in the vasodilator nerves of the cerebral arterial wall (Toda & Okamura, 1996) as well as the anococcygeus (Hobbs & Gibson, 1990). In this way NO also regulates the relaxation of smooth muscle in the gastrointestinal, urogenital and respiratory tracts (Christopherson & Bredt, 1997).

1.2.3 Endothelial nitric oxide synthase

The eNOS isoform was originally purified from bovine aortic endothelial cells (BAEC; Pollock *et al.*, 1991; Nishida *et al.*, 1992). eNOS was cloned by screening a BAEC cDNA library with primers based on the nNOS sequence (Lamas *et al.*, 1992). This revealed an open reading frame of 3.7 kilobases which had consensus sites for redox co-factors, N-terminal myristoylation and showed homology to cytochrome p450 reductase. The eNOS gene has 60% sequence identity with nNOS and 50% identity with iNOS. The human eNOS gene was later cloned and found to share 94% identity with the bovine eNOS gene with expression of eNOS mRNA predominantly in the vascular endothelium (Marsden *et al.*, 1992).

Endothelial NOS is a 135kDa protein which is located primarily in arterial and venous endothelial cells and, as with nNOS, it is constitutively expressed and its activity is regulated by physiological concentrations of Ca^{2+}. eNOS is greater than 90% particulate and this is due to post-translational myristoylation on the most N-terminal glycine residue (Lamas *et al.*, 1992; Pollock *et al.*, 1992; Sessa *et al.*, 1993). While in wild-type eNOS 95% of enzyme activity is located in the membrane fraction, when the glycine residue in position 2, which is the myristic acid acceptor site, is mutated to alanine more than 94% of eNOS activity becomes cytosolic (Sessa *et al.*, 1993). eNOS also undergoes post-translational palmitoylation on Cys^{15} and Cys^{26} which,

along with myristoylation, targets the protein to caveolae through a co-localisation with calveolin (Robinson & Michel, 1995; Shaul *et al.*, 1996; Garcia-Cardena *et al.*, 1996).

As with the nNOS isoform, activity of eNOS can be up-regulated by increases in Ca^{2+} and calmodulin binding which occurs with the activation of specific endothelial cell receptors (e.g. bradykinin B2 receptor). Furthermore, eNOS can also be activated by phosphorylation in response to shear stress (Kuchan & Frangos, 1994). Phosphorylation increases NO production by eNOS independently of Ca^{2+} levels, possibly by changing the structure of the enzyme and facilitating calmodulin binding. The use of inhibitors and kinase inactive mutants has demonstrated that phosphorylation occurs through activation of phosphatidylinositol-3-OH kinase (PI-3K) which leads to the phosphorylation and activation of the serine/threonine kinase Akt/Protein Kinase B. It appears that this kinase phosphorylates eNOS on a C-terminal residue, Ser^{1179} (bovine eNOS) or Ser^{1177} (human eNOS) resulting in enhanced NO production. Phosphorylation by Akt is specific for the membrane-anchored eNOS and does not occur with the soluble nNOS or iNOS isoforms (Fulton *et al.*, 1999; Dimmeler *et al.*, 1999). It appears that the introduction of a negative charge by phosphorylation may activate eNOS by increasing electron flow through the reductase domain (McCabe *et al.*, 2000).

1.2.4 Inducible nitric oxide synthase

Before the link between EDRF and NO was established, it was thought that mammals were incapable of producing a toxic molecule such as NO. However, the urine of subjects fed a low nitrate diet contains higher levels of nitrate than has been ingested. Thus, nitrate (and nitrite) appears to be synthesised endogenously in humans (Tannenbaum *et al.*, 1978). It was believed that this originated from the metabolism of intestinal microbes but this was proved to be incorrect by similar studies carried out using rats under germ-free conditions. Both germ-free and conventional animals excrete 10-fold more urinary nitrate than they ingest, indicating that nitrate synthesis is a mammalian process (Green *et al.*, 1981). Subsequently, it was found that rats exposed to *Escherichia coli* (*E. coli*) Lipopolysaccharide (LPS) have a 9-fold increase

in urinary nitrate excretion with the degree of enhancement of nitrate production correlating with the extent of fever (Wagner *et al.*, 1983). It was then demonstrated that the major source of urinary nitrate in LPS-treated rats is the macrophage (Stuehr & Marletta, 1985). Moreover, the cytotoxic ability of activated macrophages was shown to be dependent on the presence of L-arginine and cells produce L-citrulline and nitrite in quantities proportional to the initial concentration of L-arginine added (Hibbs, *et al.*, 1987).

From these findings and alongside the discovery of the L-arginine-NO pathway in the vasculature, it became apparent that macrophages are capable of synthesising NO as part of the antimicrobial and tumouricidal immune response. The NOS isoform responsible was first isolated from LPS and Interferon-gamma (IFN-γ)-treated murine macrophages (RAW 264.7 cells; Stuehr *et al.*, 1991b). The enzyme has a molecular weight of 125-135kDa and differs from the constitutive isoforms in that it is not found under resting conditions. When iNOS expression is induced the protein continues to produce NO as the enzyme does not require added Ca^{2+} or calmodulin for activity due to calmodulin tightly binding to iNOS at resting Ca^{2+} concentrations (Cho *et al.*, 1992). Therefore, once expressed, iNOS will produce much greater levels of NO than the constitutive isoforms. Indeed while the constitutive NOS isoforms can produce NO for periods of several mins, iNOS can remain present and synthesise NO for up to five days providing there is a sufficient supply of substrate (Vodovotz *et al.*, 1994). Consequently, it is believed that the constitutive isoforms are associated with physiological NO functions while iNOS is important during pathological episodes.

Although originally described in macrophages, expression of iNOS can also be seen upon stimulation of many murine, rat and human cells types including hepatocytes (Geller *et al.*, 1993), endothelial cells (Iwashina *et al.*, 1996), cardiac myocytes (Balligand *et al.*, 1994), osteoclasts (Brandi *et al.*, 1995), vascular smooth muscle cells (Nunokawa *et al.*, 1993), chondrocytes (Charles *et al.*, 1993) and astrocytes (Galea *et al.*, 1994).

The murine iNOS gene has been cloned and sequenced from RAW 264.7 murine macrophage cells stimulated with LPS and IFN-γ. It encodes a protein of 1144 amino

acids which has a molecular weight of 130kDa (Lowenstein *et al.*, 1992; Xie *et al.*, 1992). The C-terminal bears homology to cytochrome P450 reductase and the gene shares 51% sequence homology with the nNOS gene. The FAD, FMN and NADPH binding domains in the C-terminal are almost identical between nNOS and iNOS and the L-arginine and BH_4 binding domains share 80% homology. The human iNOS gene is approximately 37 kilobases in length and located on chromosome 17 (Chartrain *et al.*, 1994). Human iNOS cDNA has been isolated from hepatocytes and chondrocytes and shown to consist of 1153 amino acids with a molecular weight of 131kDa (Charles *et al.*, 1993; Geller *et al.*, 1993). Although the sequence cloned from human hepatocytes was reported to consist of 26 exons (Chartrain *et al.*, 1994) it now appears it is comprised of 27 exons with the discrepancy being that exon 12 is not a single exon of 278 base pairs but two exons of 195 and 83 base pairs (Xu *et al.*, 1996). The promoter region contains a number of elements that resemble known consensus sequences for the binding of transcription factors including γ-interferon response elements (γ-IRE), nuclear factor-κB (NF-κB) binding sites and tumour necrosis factor response elements (TNF-RE; Chartrain *et al.*, 1994). The murine gene promoter contains at least 22 sites which are homologous to the consensus binding sites for transcription factors. These include 10 copies of γ-IRE, 3 copies of γ-activated site (GAS) and 2 copies of the sites for activating protein-1 (AP-1), NF-κB, TNF-RE and nuclear factor-interleukin-6 (NFIL-6; Xie *et al.*, 1993).

The murine and human iNOS genes share 80% homology overall but only 66% homology in the 5'-flanking regions which stretch approximately 400 base pairs upstream of the transcription initiation site (Chartrain *et al.*, 1994). There is also a marked difference in the amount of 5'-untranslated region required in order for the murine or human iNOS promoters to drive transcription of a reporter gene. While a high level of LPS and IFN-γ-stimulated reporter gene expression can be observed with 1.5kbp of the proximal 5' region of the murine gene (Lowenstein *et al.*, 1993), it requires three regions in the proximal 16kbps of promoter sequence from the human gene to obtain cytokine-stimulated luciferase reporter expression (de Vera *et al.*, 1996). The differences in promoter sequences and transcription factor binding sites may explain the varying effects that are observed between induction of human and murine iNOS with LPS or cytokines. Indeed, while iNOS expression can be induced

in rodent cells by single agents, to express iNOS in human cells requires stimulation by a mixture of cytokines (Geller *et al.,* 1995; de Vera *et al.*, 1996).

Perhaps the most important transcription factor in the up-regulation of both human and murine iNOS in response to LPS or cytokines is NF-κB (Xie *et al.*, 1994; Taylor *et al.*, 1998). The murine iNOS gene contains two NF-κB consensus sequences, these are 5'-GGGATTTTCC-3' (-971 to -962), which is found in other genes which are up-regulated by NF-κB, and 5'-GGGACTCTCC-3' (-85 to -76) which is unique to iNOS and known as NF-κBd (Xie *et al.*, 1994). Studies using human liver and lung epithelial cells lines have demonstrated that the human iNOS gene has a putative NF-κB response element at -115 to -106 which differs from the murine NF-κBd site by one nucleotide (Taylor *et al.*, 1998). In contrast to the importance of this site for up-regulation of murine iNOS, the mutation of the human gene site has no effect on the cytokine-induced up-regulation of an iNOS promoter/luciferase reporter construct. Rather, 4 NF-κB elements located between -5.2 to -6.1kbps are essential for activation by interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-α; Taylor *et al.*, 1998).

The induction of iNOS with different combinations of LPS and cytokines can lead to much larger increases in expression than observed with LPS alone. Indeed, a study of iNOS promoter-luciferase reporter constructs found that LPS alone leads to a 75-fold stimulation of reporter activity while co-addition of IFN-γ increases this to 750-fold (Lowenstein *et al.*, 1993). Additionally, a wide variety of non-physiological substances have been shown to co-regulate iNOS expression including silica from mineral dust and allicin and ajoene from garlic (Chen *et al.*, 1995; Dirsch *et al.*, 1998).

The excessive production of NO from iNOS can lead to damage of host tissue if left unabated. It is therefore important that the induction and termination of NO synthesis from iNOS is tightly regulated. Although the main regulation of iNOS is at a transcriptional level, several post-translational modifications are required in order for the enzyme to be active. iNOS is synthesised as a monomer and the presence of substrate and co-factors is important in order for dimerisation and activity. The

purified iNOS subunits from LPS and IFN-γ-stimulated RAW 264.7 macrophages contain FAD, FMN and calmodulin but do not contain bound haem or BH_4. In order to observe iNOS dimerisation and NO synthesis, the presence/addition of L-arginine, BH_4 and haem is required (Baek *et al.*, 1993). BH_4 dependence has also been demonstrated in intact cells. iNOS subunits will not dimerise and NO cannot be generated in a BH_4-deficient cell line unless cells are supplemented with BH_4 (Tzeng *et al.*, 1995). The key enzyme in the synthesis of BH_4, GTP cyclohydrolase I, has also been shown to be up-regulated by LPS and cytokines with a similar time course to iNOS induction (Hattori & Gross, 1993) suggesting modulation of the supply of BH_4 may be an important mechanism of iNOS regulation.

Another mechanism by which NO production is limited is the availability of substrate. It has been demonstrated that iNOS relies on extracellular supplies of L-arginine and that activation of macrophages with LPS leads to an increased up-take of L-arginine (Bogle *et al.*, 1992). LPS-activated J774A.1 murine macrophage cells do not synthesise NO when L-arginine is omitted from the culture medium. Nitrite production is initiated only on the addition of extracellular L-arginine to the system (Assreuy & Moncada, 1992). Moreover, while a cellular L-arginine pool can be utilised by eNOS, this supply is inaccessible to iNOS (Closs *et al.*, 2000). The uptake of L-arginine involves a group of amino acid transporters known as system y^+ and one of the high affinity transport proteins within this group is the CAT 2 transporter. Recently mice have been created with a deletion mutation in the Cat 2 gene (Nicholson *et al.*, 2001). In peritoneal macrophages from Cat $2^{-/-}$ mice there is a 95% reduction in L-arginine uptake following LPS and IFN-γ stimulation. The importance of this for iNOS activity is reflected in the fact that the knockout (KO) cells generate only 8% of the NO produced by the wild type (WT) cells.

In addition to modulation of iNOS via regulation of synthesis and catalytic activity, NO production is limited by the degradation of the iNOS protein. Recently, it has been demonstrated in both iNOS-transfected HEK 293 cells and LPS-activated RAW 264.7 murine macrophages that iNOS protein is degraded by the 26S proteasome and this is distinct from effects that proteosomal degradation has on transcription of iNOS (Musial & Eissa, 2001). The use of mutant iNOS subunits indicates that this

degradation occurs irrespective of the enzyme's ability to dimerise or bind substrate, suggesting that the production of NO does not initiate or control this mechanism. This may represent an additional mechanism by which the cell can control the levels of NO production during inflammation.

There also exist a number of additional factors which inhibit iNOS expression at various levels. For example, interleukin-4 (IL-4) exerts a delayed suppressive effect on transcription of iNOS mRNA (Bogdan *et al.*, 1994) while interleukin-10 (IL-10) has an inhibitory effect on iNOS expression via blocking Tumour necrosis factor α (TNF-α) production (Bogdan & Nathan, 1993). Transforming growth factor β (TGF-β) has a potent inhibitory effect on iNOS expression and this is demonstrated by the apparent spontaneous iNOS expression which is observed in TGF-β1 KO mice (Vodovotz *et al.*, 1996); the serum levels of nitrate and nitrite are 4-fold higher than in WT animals and unstimulated iNOS mRNA and protein expression is observed. These animals develop multifocal inflammation and die from a wasting syndrome. These effects may be due indirectly to increases in the expression profiles of cytokines which are normally down-regulated by TGF-β1 such as IFN-γ, Interleukin-1 (IL-1) and TNF-α. Also, glucocorticoids have been shown to inhibit iNOS expression. LPS-stimulated iNOS expression is prevented *in vivo*, and in both primary macrophages and cell lines by dexamethasone. It has been demonstrated that this is partially due to the action of lipocortin-1 since pre-treatment with anti-lipocortin-1 antibodies blocks the inhibitory effects (Wu *et al.*, 1995; Bryant *et al.*, 1998). It has also been shown that glucocorticoids can act by preventing the transcription factor NF-κB binding to its DNA consensus sequence. This is apparent in a reduction in cytokine-stimulated NF-κB DNA binding in the presence of dexamethasone (Kleinert *et al.*, 1996). NO itself can feed back to modulate iNOS expression and activity and this will be discussed in Chapter 1.8.

1.3 ACTIONS OF NO

Nitric oxide is soluble in water but shows a 6 to 8-fold greater degree of solubility in lipids (Fukuto *et al.*, 2000). Although NO contains a single unpaired electron, and is

therefore a free radical, it is comparatively stable (Hobbs & Ignarro, 1999). Nitric oxide reacts with O_2, to form NO_2 that can then result in the production of N_2O_3 and N_2O_4 which, in aqueous solution, may decompose to form the stable metabolites nitrite and nitrate (Fukuto *et al.*, 2000). The rate of reaction between NO and O_2 is dependent on the concentration of NO squared, such that the rate increases as levels of NO rise. However, this reaction is unlikely to be significant *in vivo*, since concentrations of NO are small and NO reacts rapidly with oxyhaemoglobin to form methaemoglobin and nitrate (Beckman & Koppenol, 1996).

Alternatively, N_2O_3 and N_2O_4 can act as a source of nitrosonium ions (NO^+) which can react to nitrate tyrosine residues or modify protein thiol groups to form S-nitrosothiols (Hobbs & Ignarro, 1999). It is thought that, via the nitrosation of cysteine residues, NO can directly modulate the activity of proteins. For example the use of NO donors and site-directed mutagenisis of cysteine residues has indicated that NO is able to modify and regulate a number of signalling proteins such as $p21^{ras}$ (Lander *et al.*, 1995a), mitogen activated protein kinase (MAPK; Lander *et al.*, 1996) and c-Jun N-terminal kinase (JNK; Park *et al.*, 2000). While S-nitrosation of JNK suppresses activity, the activity of $p21^{ras}$ is stimulated, due to an increased rate of exchange GDP for GTP. Other proteins reported to be nitrosated include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is inactivated by S-nitrosation of four thiol groups (Vedia *et al.*, 1992). This results in an inhibition of the glycolytic pathway.

Previous studies have involved the use of NO donors or purified proteins and, as the cytoplasm contains high concentrations of glutathione and metals that could bind and sequester NO, it proved difficult to demonstrate endogenous S-nitrosation. However, recently the endogenous S-nitrosation of proteins by NO derived from nNOS has been demonstrated. S-nitrosation of proteins including GAPDH, creatine kinase, NMDA NR1 and NR2A subunits and actin is observed in brain extracts from $nNOS^{+/+}$ mice but not in the extracts made from $nNOS^{-/-}$ mice (Jaffrey *et al.*, 2001).

Nitric oxide also reacts with the free radical, superoxide (O_2^-), to form peroxynitrite ($ONOO^-$), which can be cytotoxic and damage surrounding tissues. $ONOO^-$ protonates to yield peroxynitrous acid (ONOOH) which then decomposes to give hydroxyl

radical ($OH^{\cdot}$) and $NO_2^{\cdot}$. Each of these species can participate in cellular damage via effects such as lipid peroxidation and oxidation of protein sulphydryl groups (Radi *et al.*, 1991a; Radi *et al.*, 1991b). The formation of $ONOO^-$ is prevented by the reaction of NO with oxyhaemoglobin and by the enzyme superoxide dismutase (SOD) which converts O_2^- to hydrogen peroxide (H_2O_2; Beckman & Koppenol, 1996). The presence of nitrotyrosine is taken as a marker of $ONOO^-$ formation and this is observed in a number of pathological conditions such as multiple sclerosis (Liu *et al.*, 2001), inflammatory bowel disease (Singer *et al.*, 1996), Alzheimer's disease (Good *et al.*, 1996) and myocardial inflammation (Kooy *et al.*, 1997). It is believed that $ONOO^-$ may interfere with cellular signalling mechanisms through the nitration of tyrosine residues which would otherwise be targets for phosphorylation. Tyrosine nitration has been reported to both inhibit and activate protein function (Brito *et al.*, 1999; Llovera *et al.*, 2001; Mallozzi *et al.*, 2001) and nitration may represent an alternative signalling pathway. A putative "nitrotyrosine denitrase" activity has been reported in that homogenates of rat spleen and lung which when incubated with nitrotyrosine containing BSA led to a decrease in nitrotyrosine (Kamisaki *et al.*, 1998).

Perhaps the most important reaction with respect to the physiological role of NO is the interaction with transition metals, in particular iron. The binding of NO to the iron in the haem group of soluble guanylate cyclase (sGC) is responsible for the activation of this enzyme and the production of the second messenger guanosine-3'5'-monophosphate (cGMP). Thus, the biological actions of NO can be classified as cGMP-dependent or -independent on the basis of whether NO acts directly or through the activation of sGC. This enzyme is a ubiquitously expressed, haem-containing protein which is a dimer of α and β subunits, both of which are necessary for catalytic activity (Hobbs & Ignarro, 1996; Hobbs, 1997). Initially it was recognised that L-arginine was an endogenous activator of guanylate cyclase identified in the supernatant of neuroblastoma cells (Deguchi & Yoshioka, 1982). It is now known that L-arginine is the precursor of NO which binds to the haem moiety of sGC leading to an up to 200-fold increase in activity of the enzyme resulting in conversion of guanosine-5'-triphosphate (GTP) to cGMP (Murad, 1994). cGMP regulates cellular function by 3 mechanisms: activation of cGMP-dependent protein kinase (G-kinase),

cyclic nucleotide-gated ion channels and modulation of cyclic nucleotide phosphodiesterases (PDEs; Hobbs & Ignarro, 1996; Hobbs, 1997).

NO can also act by binding directly to target proteins (i.e. cGMP-independent) through an interaction with the metal ions in protein subunits. As mentioned above, NO forms a complex with the Fe in the haem group of sGC causing a conformational change which up-regulates protein activity (Murad, 1994). NO can interact with the iron in the haem moieties of haemoglobin, cytochrome oxidase (Hobbs & Ignarro, 1999) and NOS itself (Assreuy *et al.*, 1993; Griscavage *et al.*, 1993; see Chapter 1.8.1).

1.4 MACROPHAGES AND NO

Macrophages are effector cells of the immune system which participate in host defence through the killing and removal of bacteria, tumour cells and other infectious stimuli. The cells originate from pluripotential stem cells in the bone marrow which, depending on the stimuli present, can differentiate into lymphocytes, erythrocytes or neutrophils as well as monocytes (Davies, 1994).

Macrophage colony stimulating factor (M-CSF) is a haematopoietic growth factor which stimulates the growth of murine bone marrow monocytic precursors. M-CSF is released by a variety of cells including fibroblasts, endothelial cells, B and T cells and monocytes/macrophages themselves. Although M-CSF can act on other cells, binding to the M-CSF receptor on monocytes leads to mitosis, secretion of cytokines, an increase in phagocytic capacity and chemotaxis. M-CSF also prepares the monocytes for response to a secondary signal (Fixe & Praloran, 1998).

The presence of IL-1, interleukin-3 (IL-3) and interleukin-6 (IL-6) will initiate differentiation of the stem cell. If the pluripotent myeloid precursor is further exposed to IL-1 and IL-3 then a granulocyte/monocyte precursor is formed. In the presence of M-CSF, granulocyte macrophage colony stimulating factor (GM-CSF) and IL-3 the granulocyte/monocyte precursor becomes a monocyte precursor (Valledor *et al.*, 1998). Cells committed to the monocyte lineage go through further rounds of

differentiation, from monoblast to promonocyte and finally to monocytes. Monocytes have developed lysosomal systems and strong phagocytic ability and generally have lost their ability to proliferate (Gordon, 1999). Once released into the blood stream the monocytes migrate to tissues where they undergo morphological and functional differentiation to perform specific functions. The peripheral blood monocytes differentiate into macrophages in tissue and have increased amounts of lysosomes, hydrolytic enzymes and mitochondria (Davies, 1994). The exact function of the macrophage will depend on the tissue, and specific types include Kupffer cells in the liver, alveolar macrophages in the lung, osteoclasts in bone and peritoneal macrophages in the serous cavities. These cells have a half life of between 60 and 90 days, although this is reduced when they encounter inflammatory stimuli (Davies, 1994). The presence of inflammatory stimuli leads to the recruitment of macrophages which localise to specific sites by the binding of adhesion molecules and chemotaxis (Gordon, 1999).

The macrophages perform their host defence role by a number of methods with the aim of killing the infectious agent without damage to the macrophage host tissue. These include phagocytosis of particulate stimuli, which are recognised by their coating of antibodies or opsonins (Greenberg, 1999). Phagocytosis begins by an invagination of the macrophage plasma membrane and the formation of pseudopodia which wrap around the stimuli to form phagocytic vacuoles. These fuse with lysosomes which are rich in proteinases, glycosidases and lipases that degrade the antigen. The macrophage has the ability to present partially degraded antigen molecules to T lymphocytes.

Macrophages also kill bacteria and tumour cells via the synthesis and secretion of biological substances that facilitate removal of pathogenic stimuli and wound repair and healing. Among the molecules secreted upon macrophage activation are a number of cytokines (Sundy *et al.*, 1999). The cytokine IL-1 stimulates the production of prostaglandins and neutral proteinases as well as the synthesis of other cytokines such as IL-6 and TNF-α. TNF-α is involved in tumour cell killing and IL-6 aids the growth of haematopoietic progenitors, the expansion of T-lymphocyte clones and enhances B-lymphocyte antibody production. While these three cytokines are pro-

inflammatory, macrophages synthesise and release IL-10 which acts as a negative feedback to down-regulate inflammation. Macrophages also synthesise and secrete hydrolytic enzymes such as collagenase which regulate connective tissue turnover. Also, activated macrophages produce lipid mediators including the prostaglandins; PGE_2, PGI_2 and thromboxane A_2 and the leukotrienes LTB_4 and LTC_4 (Katori & Majima, 2000). The encountering of inflammatory stimuli leads to a metabolic burst by the macrophage and this results in the production of oxygen metabolites. This includes activation of the enzyme NADPH oxidase which reduces O_2 to produce O_2^- (Morel *et al.*, 1991). This can act alone, or react with NO to form $ONOO^-$, to exert cytotoxicity.

The activation of macrophages by cytokines, bacterial products and tumour cells also leads to the expression of iNOS and the production of NO, which acts as a cytotoxic and cytostatic effector molecule (MacMicking *et al.*, 1997). The cytotoxic and cytostatic actions of NO are mediated by inhibition of key cellular enzymes within the target cells. For example, NO can reversibly inhibit complex IV of the mitochondrial respiratory chain in competition with O_2 and irreversibly inhibit complex I (Brown, 1995; Lizasoain *et al.*, 1996; Clementi *et al.*, 1998). Furthermore, NO inhibits cellular metabolism through inhibition of the enzyme aconitase which forms part of the citric acid cycle (Hibbs, *et al.*, 1988). S-nitrosation of GAPDH on 4 cysteine residues also occurs which inactivates the enzyme and can inhibit glycolysis (Vedia *et al.*, 1992).

The release of NO by macrophages, as well as defending against invasion by micro-organisms, prevents the growth of tumour cells. These cells proliferate rapidly and thus require a constant rate of DNA synthesis. The rate-limiting enzyme in DNA synthesis is ribonucleotide reductase, which converts ribonucleotides to deoxyribonucleotides. This enzyme is also targeted by NO, with exposure to NO donors causing a concentration-dependent inhibition of activity, possibly by interacting with a tyrosyl radical on the R2 subunit which is necessary for activity (Lepoivre *et al.*, 1991). As well as binding key enzymes and modifying activity, NO can also have a toxic effect by acting directly on genomic DNA. Exposure to NO has been shown to cause deamination of DNA which can lead to depurination, mispairing and strand breaks. This mutagenic effect has been observed in bacteria as well as mammalian cells (Wink *et al.*, 1991; Nguyen *et al.*, 1992).

1.5 NUCLEAR FACTOR-KAPPA B (NF-κB)

1.5.1 NF-κB

The transcription factor NF-κB is probably the most important regulator of iNOS expression. NF-κB was first identified as a nuclear factor that bound to the promoter of the Immunoglobulin κ light chain gene in B-cells (Sen & Baltimore, 1986), but was subsequently found to be ubiquitously expressed in the cytoplasm of most cells. Under resting conditions NF-κB is sequestered in the cytosol due to the binding of an inhibitory protein, IκB, but upon activation, NF-κB translocates to the nucleus where it activates the transcription of a wide range of pro-inflammatory proteins (Baeuerle & Baltimore, 1988a; Baeuerle & Baltimore, 1988b; Figure 2). As activation of NF-κB does not require *de novo* protein synthesis, this represents a rapid way in which factors involved in the immune response can be up-regulated.

Stimuli which lead to activation of NF-κB include cellular damage caused by reactive oxygen intermediates (ROIs), hypoxia, ultra-violet light, infection and the presence of LPS, viral-transactivating proteins, double-stranded RNA and cytokines (including IL-1 and TNF-α; Baeuerle, 1991; Ghosh *et al.*, 1998). Certain factors known to inhibit NF-κB include IL-10 (Bogdan & Nathan, 1993) and glucocorticoids, which can inactivate NF-κB by inhibiting DNA binding (Kleinert *et al.*, 1996).

NF-κB is a heterodimer which is formed from members of the Rel family of proteins, so called because they share a 300 amino acid region known as the Rel homology domain. This domain is responsible for DNA-binding, dimerisation, interaction with IκB proteins and also contains the nuclear localisation sequence (NLS). The members of this family include c-Rel, p65, p100/p52, p105/p50 and also the *Drosophila* proteins dorsal, dif and relish (Ghosh *et al.*, 1998). The C-terminal regions of p100 and p105 share homology with the IκB family of proteins and thus act to sequester NF-κB dimers in the cytosol. These proteins are proteolytically cleaved to produce p52 and p50 respectively (Fan & Maniatis, 1991). Only certain combinations of Rel proteins are found, each with a different transactivating potential. p50/p65, p50/c-rel,

p65/c-rel and p65 homodimers all lead to transcriptional activation upon DNA-binding but p50 or p52 homodimers are repressive (Ghosh *et al.*, 1998). Transgenic animals have been created where different members of the Rel family have been knocked out. p50 KO mice show no developmental abnormalities but defects in immune responses are observed. These include reduced B-cell antibody production and altered susceptibility to bacterial and viral infection (Sha *et al.*, 1995). p65 KOs die at day 16 of gestation due to massive liver degeneration by apoptosis, indicating that p65 has a critical role in development. Embryonic fibroblasts from these animals are defective in TNF-α stimulation of IκBα mRNA production but basal levels are unaltered, suggesting p65 controls inducible transcription (Beg *et al.*, 1995a).

The consensus κB binding site is in the form 5'-GGGGYNNCCY-3'. As two Rel proteins each make up half of the DNA binding site this allows for variations in the 10 base pair consensus site. There are a wide range of proteins which contain κB binding sites in their promoter and these include adhesion molecules (vascular cell adhesion molecule-1; VCAM-1, intracellular adhesion molecule-1; ICAM-1), cytokines (IL-1, IL-2, IL-6, interleukin-8 (IL-8), TNF-α, tumour necrosis factor-beta (TNF-β) and interferon-beta (IFN-β)), iNOS, cyclo-oxygenase-2 (COX-2) and acute phase response proteins including angiotensin and complement proteins (Baldwin, 1996).

1.5.2 IκB

IκB masks the NLS of NF-κB and retains the transcription factor in the cytoplasm. Cellular activation by stimuli mentioned previously leads to phosphorylation of IκB followed by ubiquitination and degradation by the 26S proteasome complex allowing nuclear translocation of NF-κB and gene transcription. The IκB proteins are a family made up of IκBα, β, ε, γ, Bcl-3 and the *Drosophila* protein cactus. Each family member has multiple copies of a 33 amino acid region known as the ankyrin repeat which mediates protein-protein interactions with Rel homology domains (Karin & Ben Neriah, 2000).

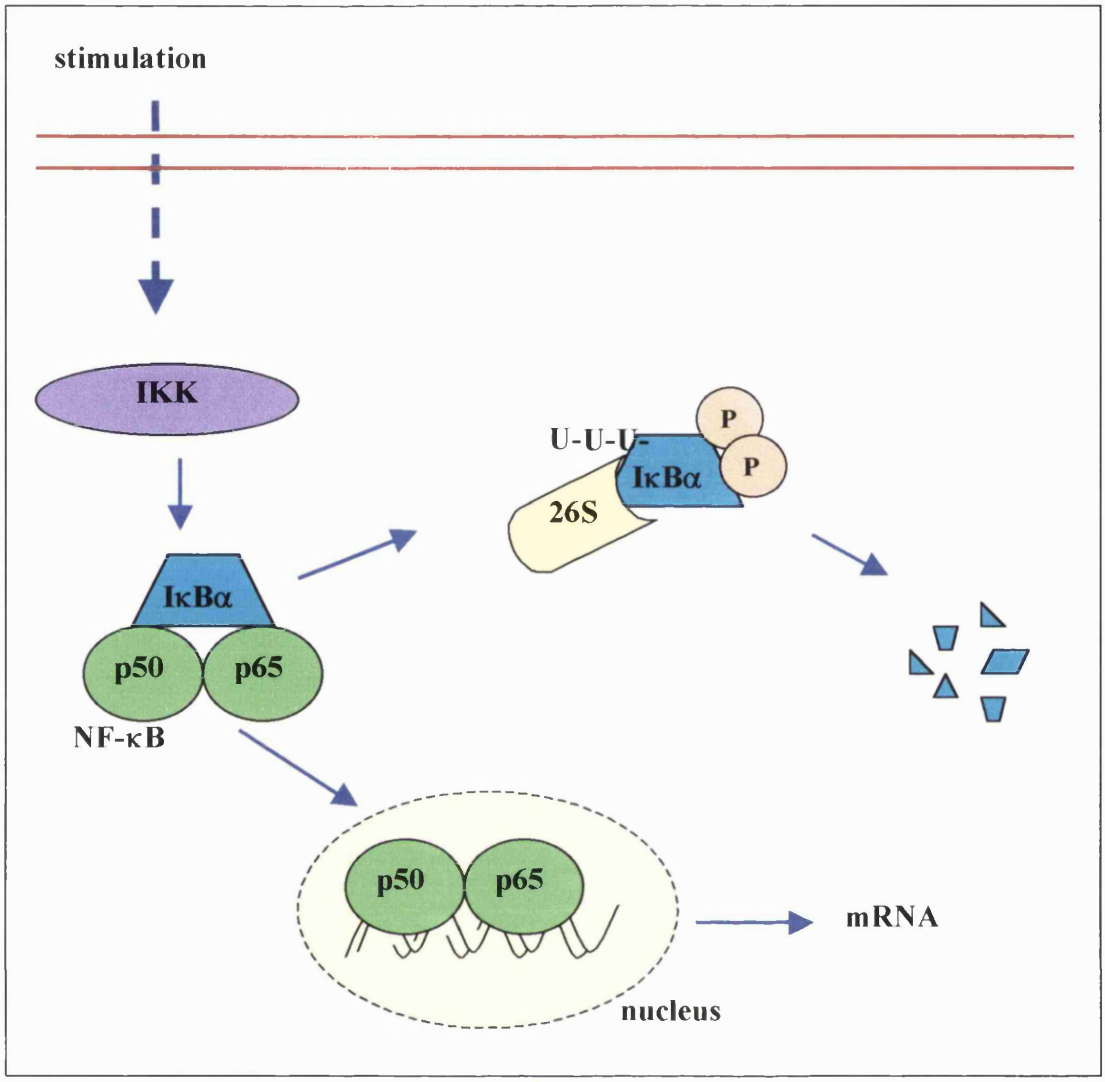


Figure 2: Stimulation of IKK and activation of NF-κB. IKK – IκB kinase; IκBα - inhibitor of κB α; P – phosphorylation; U – ubiquitin; 26S – 26S proteasome complex; p50 – NF-κB p50 subunit; p65 – NF-κB p65 subunit

The IκBα protein was the first to be cloned (Davis *et al.*, 1991; Haskill *et al.*, 1991) and thus many studies have been carried out on this isoform. IκBα is a 37 kDa protein with the N-terminal 70 amino acids being phosphorylated in response to signals, the central 205 amino acids containing the ankyrin repeats and the C-terminal 42 amino acids including a domain which is responsible for basal turnover of the protein (Baldwin, 1996). IκBα is rapidly degraded after phosphorylation and its removal leads to a transient activation of NF-κB. The promoter of IκBα contains a κB consensus sequence and thus an autoregulatory feedback loop exists by which IκBα is degraded and then subsequently re-synthesised via NF-κB activation (Sun *et al.*, 1993; Chiao *et al.*, 1994). The newly synthesised IκBα can bind to and inactivate NF-

κB; indeed it has recently been discovered that residues 45-54 of IκBα encode a nuclear export sequence which allows the cytoplasmic localization of the inactive complex (Huang *et al.*, 2000).

IκBβ is a 45kDa protein and its degradation leads to a more persistent activation of NF-κB as, unlike IκBα, it is not up-regulated by this transcription factor. IκBβ levels remain low until the activating signal has been attenuated (Thompson *et al.*, 1995). The creation of chimeric IκBs has shown that IκBα forms a stronger bond with NF-κB in the cytoplasm than IκBβ due to a β-turn motif in its first ankyrin repeat (Simeonidis *et al.*, 1999).

The majority of NF-κB heterodimers are formed from p50/p65 or p50/c-Rel and are regulated by IκBα or IκBβ. All known inducers of NF-κB lead to degradation of IκBα, while only a subset of activators, including LPS and IL-1, also promote the degradation of IκBβ (Thompson *et al.*, 1995). The other members of the IκB family have more limited functions due to their preferences for particular Rel proteins or tissue-specific expression. The phosphorylation of IκBα has been investigated in HeLa cells transfected with IκBα and stimulated with okadaic acid. The removal of the C-terminal amino acids has no effect on phosphorylation while deletion of the N-terminal blocks IκBα phosphorylation. There are 3 hydroxy amino acids in the N-terminal: Ser^{32}, Ser^{36} and Tyr^{42}, which are highly conserved across species. $Ser^{32}\rightarrow$Ala and $Ser^{36}\rightarrow$Ala mutants lead to a reduction in phosphorylation while mutation of both of these serine residues concomitantly abolishes phosphorylation (Traenckner *et al.*, 1995). Therefore, IκBα appears to be phosphorylated on Ser^{32} and Ser^{36} in the N-terminal before proteasomal degradation. These residues are conserved across isoforms and represent Ser^{19} and Ser^{23} on IκBβ. Following phosphorylation ubiquitin molecules are attached to Lys^{21} and Lys^{22} in the N-terminal of IκBα.

Studies using proteasome inhibitors have demonstrated that IκB is phosphorylated and degraded before dissociation from NF-κB occurs. It was found that IκBα is degraded after stimulation with phorbol myristate, IL-1, LPS and TNF-α and this coincides with the appearance of NF-κB in the nucleus (Henkel *et al.*, 1993). In

human cell lines the cleavage of p105 to produce p50 requires the presence of ubiquitin and ATP and this, as well as IκBα degradation, is blocked by inhibitors of the 26S proteasome complex (Palombella *et al.*, 1994).

IκBα KO mice have been generated and while these animals appear normal at birth they die within 9 days (Beg *et al.*, 1995b). This is due to a rapid physiological degeneration including atrophy of the spleen and thymus, severe runting and development of skin abnormalities. An elevated level of nuclear NF-κB is observed in haematopoietic cells from these animals, but only mRNA levels of certain genes are increased, indicating the need for other factors. In the embryonic fibroblasts, treatment with TNF-α leads to no differences in NF-κB activation between WT and KO cells indicating the importance of cytoplasmic IκBβ in inhibition of the transcription factor. Despite this, IκBα appears to be important for the cessation of NF-κB activity with nuclear levels of the transcription factor maintained in its absence. The differences in NF-κB activation between the above cell types from $I\kappa B\alpha^{-/-}$ animals indicate that the importance of each IκB isoform is cell-type specific although IκBα is necessary to down-regulate NF-κB.

1.5.3 IκB Kinase

The phosphorylation of IκB is carried out by the IκB kinase (IKK). IKK was originally isolated as a 700-900kDa complex, consisting of multiple specific IKK subunits. IKKα (85kDa) and IKKβ (87kDa) have been identified as the catalytic subunits of the complex (these subunits are also referred to as IKK1 and IKK2; DiDonato *et al.*, 1997; Mercurio *et al.*, 1997). Each of these subunits has a N-terminal protein kinase domain which is Ser/Thr specific. A third subunit, IKKγ or NF-kappaB essential modulator (NEMO) has also been identified; this 48kDa protein does not appear to have catalytic activity and function as a regulatory subunit (Rothwarf *et al.*, 1998).

IKK has been found in human cells lines to consist of IKKα:IKKβ heterodimers in association with dimers or trimers of IKKγ (Rothwarf *et al.*, 1998). IKKβ associates

with IKKγ, which links the complex to upstream activators. IKK is transiently activated, by phosphorylation, and preferentially phosphorylates IκB subunits which are complexed with NF-κB, over free IκBs. The study of TNF-α stimulated HeLa cells showed that mutation of Ser^{177} and Ser^{181} on the IKKβ subunit abolishes IKK activation while the equivalent mutation of IKKα has no effect (Delhase *et al.*, 1999). Once IKKβ is activated, the subunit undergoes progressive autophosphorylation at C-terminal serine residues which de-activate the complex.

The creation of transgenic mice has helped further identify the specific functions of the IKKα and β subunits. Mice which have the IKKα gene knocked out die within several hours of birth due to defects in dermal and skeletal development (Hu *et al.*, 1999; Takeda *et al.*, 1999). Despite this, embryonic fibroblasts cultured from these KOs show normal activation of IKK, rapid degradation of IκBα and nuclear translocation of NF-κB. $IKK\beta^{-/-}$ mice die between embryonic day 12.5 and 14 due to liver degeneration resulting from hepatocyte apoptosis (in a similar manner to that observed for p65 KO mice; Li *et al.*, 1999). The activation of $IKK\beta^{-/-}$ embryonic fibroblasts with TNFα and IL-1 results in an increased stability of IκBα and β and a reduction in NF-κB activity, as compared to WT cells. This illustrates that IκBβ is the key catalytic subunit of IKK.

1.6 ACTIVATION OF NF-κB

1.6.1 LPS-induced NF-κB activation

LPS is an outer membrane constituent of gram-negative bacteria such as *E. coli*. It is a complex glycolipid composed of hydrophilic polysaccharides and a hydrophobic domain known as Lipid A, which is a common component in the LPS from different bacteria. Diversity of structures is determined by the O-antigens in the molecule core (Guha & Mackman, 2001).

The lipid A component of LPS rapidly binds to a serum binding protein found in the bloodstream called LPS binding protein (LBP), a 60kDa glycoprotein that is

synthesised by hepatocytes. LBP is present in serum at concentrations <0.5μg/ml but 24 hours after induction of the acute phase response levels can rise to 50μg/ml. The presence of LBP enhances LPS-induced TNF-α production whereas no increase in TNF-α is observed with LPS alone (Schumann *et al.*, 1990). The LPS-LBP complex is then recognised by an antigen which has both soluble and membrane-bound forms, CD14 (Guha & Mackman, 2001). CD14 is a 55kDa glycoprotein which can be found in serum or attached to the outer leaflet of the monocyte cell membrane via a glycophosphatidylinositol anchor. The blockade of CD14 with monoclonal antibodies prevents LPS-stimulated TNF-α synthesis in whole blood (Wright *et al.*, 1990). While it is accepted that LPS binds to CD14 on the surface of monocytes, it is also apparent that CD14 lacks a transmembrane domain. Thus, the mechanism by which CD14 transduces the signal to the cytoplasm was originally unclear. This uncertainty was clarified by comparison to a similar system found in *Drosophila*. Members of the Rel/NF-κB family of transcription factors found in *Drosophila* are know as dorsal, dif and relish (Ghosh *et al.*, 1998). Under resting conditions these proteins are held by the IκB homologue, cactus, but degradation of cactus occurs through the activation of a transmembrane receptor known as toll. This receptor is activated by a ligand called spatzle and is involved in dorsal-ventral patterning in embryos and also in the induction of an anti-fungal response (O'Neill, 2000). Toll shares homology with the IL-1 receptor (IL-1R) and part of this conserved sequence was used to search human cDNA libraries to identify homologous genes which may initiate NF-κB activation in mammalian cells (Medzhitov *et al.*, 1997). A 2523 bp open reading frame was cloned and found to produce an 841 amino acid protein, termed toll-like receptor (TLR). TLR mRNA was found in monocytes, macrophages, dendritic cells and T-cells. A family of human TLRs has now been cloned with at least 10 members (TLR1-10), each containing an extracellular domain with leucine-rich repeats and a cytoplasmic domain bearing homology to the IL-1R cytoplasmic domain (Rock *et al.*, 1998; Akira *et al.*, 2001).

TLR-2 and -4 are highly expressed in monocytes and several observations confirm the importance of each isoform in the response to LPS. Mice which have a mis-sense mutation in the TLR-4 gene leading to Pro^{712} being replaced by His, and animals that are homozygous for a TLR-4 null mutation (Poltorak *et al.*, 1998), both exhibit

resistance to LPS. The role of TLR-4 in LPS signalling has also been confirmed by examining the effects of LPS treatment of WT, TLR-$2^{-/-}$ and TLR-$4^{-/-}$ mice. While only a fifth of the WT or TLR-2 KOs survive on day 6 post-injection, all of the TLR-4 KOs are still alive suggesting that this receptor is involved in LPS signalling. Furthermore, in LPS-treated peritoneal macrophages from each of these strains, cytokine synthesis is only observed in WT or TLR-2 KO mice. In contrast, stimulation of macrophages with cell wall extracts from *Staphylococcus aureus* leads to TNF-α production in WT and TLR-4 KOs, but not in TLR-2 KOs (Takeuchi *et al.*, 1999). Therefore, it appears that TLR-4 is the transmembrane component of the LPS receptor while TLR-2 is involved in the response to Gram positive bacteria.

In order to link TLR-4 with the activation of NF-κB, HEK 293 cells were transfected with TLR-4 cDNA and a NF-κB-driven promoter-luciferase reporter construct (Chow *et al.*, 1999). The transfection of TLR-4 alone, or stimulation of transfected cells, leads to an increase in luciferase activity. Stimulation with LPS and soluble CD14 leads to a much larger increase in luciferase activity but this is only observed in the presence of TLR-4, indicating an interaction between all three molecules is required for NF-κB activation (Chow *et al.*, 1999).

Recent studies have indicated that the LPS-binding complex also requires another protein known as MD-2 (Shimazu *et al.*, 1999). Cross-linking studies in HEK 293 cells show that LPS only comes into close proximity with TLR-4 when it is part of a LPS-CD14 complex and this association also requires the presence of MD-2 (da Silva *et al.*, 2001). Therefore, it appears that LPS binds to the surface of the monocyte via an interaction with both CD14 and TLR-4 (in the presence of MD-2), with the TLR-4 component of the LPS-binding complex transmitting the signal across the cell membrane (see Figure 3).

The intracellular signalling pathway by which LPS activates NF-κB has not only been elucidated by its homology to signalling pathways observed in *Drosophila*, but also by its similarity to the IL-1-induced pathway. In *Drosophila* a protein known as tube binds to the intracellular domain of the toll receptor (Akira *et al.*, 2001). A human homologue of tube, called MyD88, interacts with the same domain on the IL-1R.

MyD88 was originally identified as a myeloid differentiation marker but was subsequently found to co-precipitate with IL-1R in an IL-1-dependent manner (Wesche *et al.*, 1997). It has been found that MyD88 immunoprecipitates bound to TLR (Medzhitov *et al.*, 1998). MyD88 KO mice do not develop endotoxic shock in response to high doses of LPS, as is seen in WT animals, and their macrophages produce no IL-6 or TNFα and only low levels of nitrate (Kawai *et al.*, 1999). Therefore, it appears that MyD88 is necessary for LPS-signalling, probably through its interaction with TLR. Recently, another TLR adaptor protein has been identified and termed MyD88-adapter-like (Mal) due to its homology with MyD88, although Mal appears to be specific for TLR-4 mediated activation of NF-κB (Fitzgerald *et al.*, 2001).

Studies of IL-1 receptor signalling have demonstrated that MyD88 acts as an adaptor protein to link the receptor to the IL-1 receptor associated kinase (IRAK). In experiments using the human pro-monocytic THP-1 cell line, the kinase activity of endogenous IRAK is activated in response to LPS with stimulation leading to the association of IRAK with MyD88 (Li *et al.*, 2000). A role for IRAK in LPS-induced signalling is confirmed by looking at the administration of high doses of LPS to IRAK WT and KO mice. LPS proves lethal to 40% of WT animals but only 19% of KO animals die (Swantek *et al.*, 2000).

Downstream of IRAK is the protein tumour necrosis factor receptor-associated factor 6 (TRAF-6), which interacts with IRAK. TRAF-6 links the upstream molecules to the mitogen activated protein kinase kinase kinases (MAP3Ks) which are responsible for phosphorylating and activating the IKK complex. Dominant negative mutants of TRAF-6 show impaired LPS-stimulated iNOS expression and NF-κB activation (Lomaga *et al.*, 1999).

Another molecule, TGF-β-activated kinase (TAK-1) has recently been identified as being necessary for LPS stimulated activation of NF-κB (Irie *et al.*, 2000). It appears that this MAP3K family member functions downstream of MyD88 and TRAF-6, as part of a protein kinase complex termed TRAF-6-regulated IKK activator 2

(TRIKA2). TAK-1 appears to be a kinase which acts downstream of TRAF-6 to phosphorylate the IKK complex (Wang *et al.*, 2001)

Several other MAP3Ks have been identified which phosphorylate IKK on specific serine residues leading to activation, phosphorylation of IκB and nuclear translocation of NF-κB. These are MAP3K-1, 2 and 3 (also known as MEK-1, 2 and 3) and NF-κB inducing kinase (NIK). MEK1 and NIK can activate the IKK complex in parallel, possibly leading to synergistic activation of the complex (Nakano *et al.*, 1998; Nemoto *et al.*, 1998). NIK appears to interact with most TRAF proteins, regardless of their ability to activate NF-κB (Baud *et al.*, 1999).

Despite these recent advances, the pathway(s) of LPS-induced activation of NF-κB are still incompletely understood, with additional signalling molecules being identified which play a pivotal role. One such molecule is $p21^{ras}$. In rat primary astrocytes, transfection with a dominant negative mutant of $p21^{ras}$, where Ser^{17} has been mutated to Asp, leads to an inhibition of LPS-induced GTP-loading of $p21^{ras}$ and results in an inhibition of NF-κB DNA-binding activity and subsequent NO production (Pahan *et al.*, 2000). It has been suggested that $p21^{ras}$ may fit into the previously described signalling pathway through an interaction with TRAF-6. In human fibroblasts TRAF-6-stimulated activation of an IL-8- or NF-κB-promoter-reporter construct is blocked by a dominant negative $p21^{ras}$ mutant (Caunt *et al.*, 2001).

Although these pathways apply to monocytes and macrophages, non-myeloid cells such as vascular smooth muscle cells do not express a known receptor for LPS, indicating alternative pathways are also important in activation of NF-κB. In both RAW 264.7 macrophages and primary cultures of rat aortic smooth muscle cells it has been demonstrated that stimulation by LPS and up-regulation of iNOS expression involves the activation of members of the protein kinase C (PKC) family (Paul *et al.*, 1997). The use of inhibitors directed at specific MAPK isoforms illustrates that, while p44/42 MAPK and p38 MAPK are activated by LPS in macrophages, only p38 is required for LPS-induced iNOS induction (Chen & Wang, 1999). The transfection of HeLa and HEK 293 cells with IRAK or TRAF-6 expression vectors demonstrates that

both these proteins activate JNK and p38. Co-expression of both IRAK and TRAF-6 does not result in a greater activation than with either protein alone, suggesting a common pathway for the activation of these MAPK isoforms (Baud *et al.*, 1999).

Pathways leading to LPS-induced NF-κB activation are summarised in Figure 3.

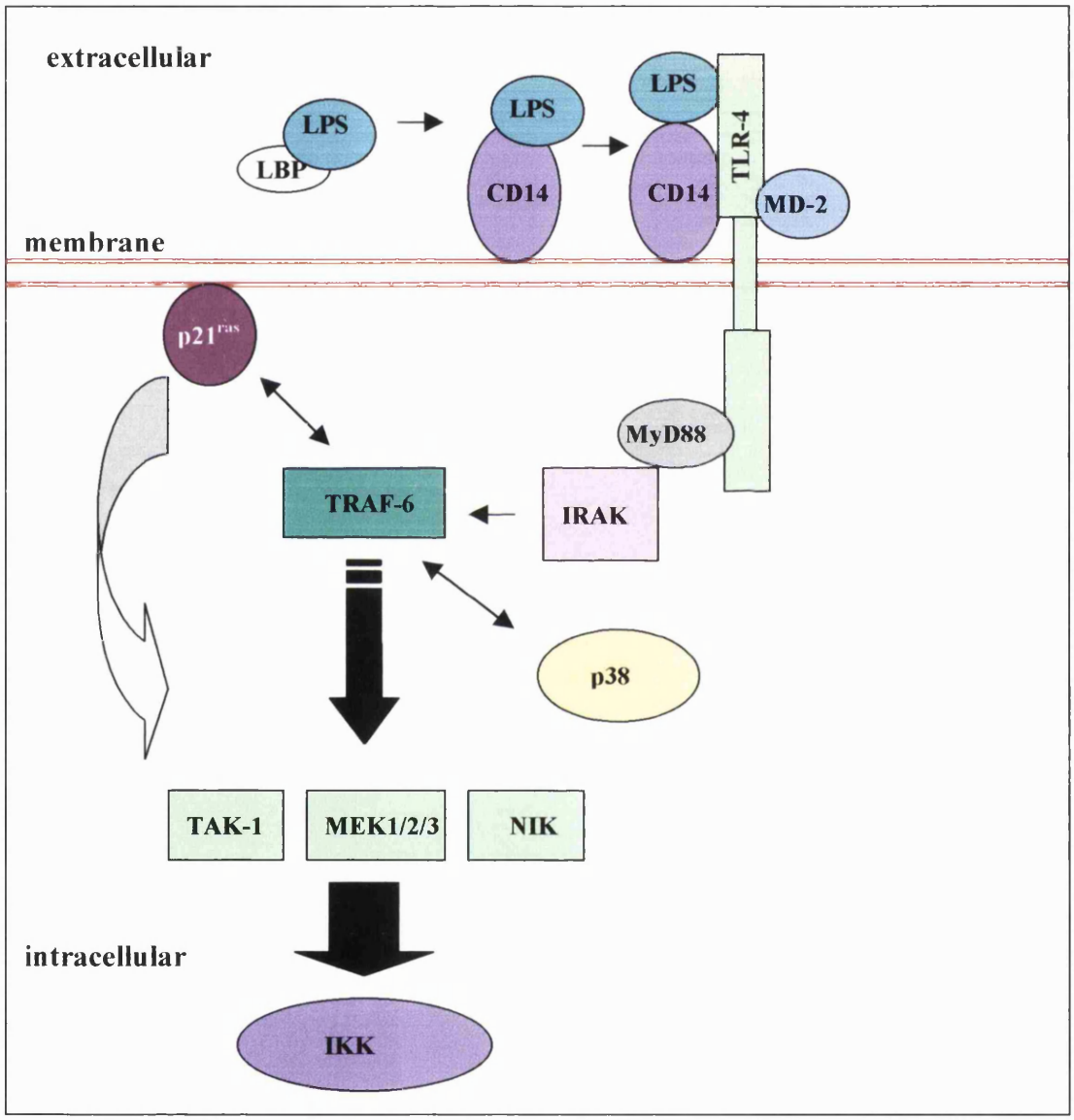


FIGURE 3. LPS-stimulated activation of NF-κB. LPS – lipopolysaccharide; LBP – LPS binding protein; TLR-4 – toll like receptor 4; IRAK – IL-1 receptor associated kinase; TRAF-6 – tumour necrosis factor receptor-associated factor 6; p38 – p38 mitogen activated protein kinase; TAK-1 – TGF-β-activated kinase; MEK – mitogen activated protein kinase kinase kinase; NIK – NF-κB inducing kinase; IKK – IκB kinase

1.6.2 Other activators of NF-κB

The pathway described previously illustrates the factors involved in the activation of NF-κB by LPS. The activation of the macrophage by LPS also leads to the production TNF-α which participates in a positive feedback to further up-regulate NF-κB, and therefore appears to play a central role in mounting the inflammatory response induced by LPS.

TNF-α binds to the membrane receptor TNF-receptor I which is ubiquitously expressed and mediates most TNF-α functions via its cytoplasmic 'death domain'. This interacts with a number of molecules including TRAF-2, which can activate NIK leading to IKK activation and degradation of IκB (Baud *et al.*, 1999). Although LPS, TNF-α and IL-1 can activate NF-κB and subsequent protein expression alone, a synergistic effect is observed in the up-regulation of proteins such as iNOS when they are combined with IFN-γ. IFN-γ is secreted by Th_1 cells and acts through the transcription factors signal transducer and activator of transcription 1 (STAT1) and interferon regulatory factor 1 (IRF-1). The binding of IFN-γ causes receptor dimerisation/oligomerisation and the activation of janus kinase (JAK) 1 and 2. These in turn activate STAT1 which is released from the membrane and translocates to the nucleus where it binds to the γ-activated sequence (GAS; Briscoe *et al.*, 1996). The promoter for IRF-1 contains a GAS element and is therefore induced by STAT1 binding.

The promoter of the murine iNOS gene contains NF-κB response elements, a GAS element and an γ-IRE (Lowenstein *et al.*, 1993; Xie *et al.*, 1994). A recent study was carried out to investigate the mechanism by which IFN-γ and TNF-α synergise in their activation of iNOS expression in RAW 264.7 murine macrophages (Saura *et al.*, 1999). While both factors alone induce expression of iNOS mRNA, a strong synergistic effect of at least 5-fold higher induction is observed with TNF-α and IFN-γ together. It appears that this effect is due to an interaction between the transcription factors NF-κB and IRF-1, which is demonstrated through their co-precipitation from either a γ-IRE or NF-κB site after activation. Nuclear extracts from cells stimulated

with both TNF-α and IFN-γ can also promote rapid cyclisation of a piece of linear DNA consisting of a section of the iNOS promoter where the γ-IRE is 900 base pairs upstream of the NF-κB site (Saura *et al.*, 1999). Therefore it seems that these transcription factors interact and bend the intervening DNA, which may increase the efficiency of recruitment of basal transcription factors. Recently it has been discovered that the human iNOS promoter contains overlapping NF-κB and STAT-1 elements at -5.8kb, which suggests a site for co-operative binding and synergy between these two transcription factors (Ganster *et al.*, 2001).

1.6.3 Regulation of NF-κB by reactive oxygen intermediates

There is a large amount of evidence to suggest that the activity of NF-κB is modulated by ROIs. These species are produced when the incomplete reduction of oxygen occurs during respiration or during inflammatory/pathological episodes. The presence of excessive levels of such molecules, including O_2^-, H_2O_2 and OH^-, constitutes a situation referred to as oxidative stress. Factors which induce oxidative stress include UV light, the oxidative burst produced by immune cells, inflammatory cytokines and hypoxia. The cell possesses a number of mechanisms with which to deal with the production of ROIs such as the enzyme SOD, which converts O_2^- to H_2O_2, and catalase, which breaks H_2O_2 down to H_2O and O_2. The molecules N-acetyl cysteine (NAC) and reduced glutathione (GSH) are present in the cytosol and act as scavengers of ROIs. Oxidative stress occurs when these defence mechanisms cannot cope with levels of ROIs.

There are several findings which indicate a role for ROIs in the activation of NF-κB (Bowie & O'Neill, 2000). First, there is a direct effect of ROIs on NF-κB activity. H_2O_2, activators of the macrophage respiratory burst, adenosine diphosphate (ADP) and phorbol myristic acid (PMA) all lead to NF-κB activation in J774.1 and rat alveolar macrophages (Kaul & Forman, 1996). Second, the inhibition or over expression of enzymes that modulate ROI levels affect NF-κB activity. In cells which over express catalase or SOD, TNFα-induced activation of NF-κB is impaired or potentiated, respectively (Schmidt *et al.*, 1995). As catalase breaks down H_2O_2 and

SOD increases H_2O_2 levels this would suggest that this ROI mediates TNF-α-induced activation of NF-κB. Third, it has also been demonstrated that compounds which act as antioxidants inhibit activation of NF-κB. The antioxidant pyrrolidine dithiocarbamate (PDTC) blocks LPS-induced NF-κB activation in RAW 264.7 macrophages (Xie *et al.*, 1994). In HeLa cells that are stimulated with TNF-α, NF-κB DNA binding activity is reduced by pre-incubation with NAC (Li & Karin, 1999). IκBα degradation is partially blocked, yet IKK activity is not affected, suggesting an action of ROIs on the mechanism of degradation.

In contrast, there have been a number of studies which have found that changes in ROI levels do not have an effect on NF-κB activity, possibly because such effects may be cell-type specific. One difference between individual cell types may be the intracellular levels of GSH, with higher levels preventing effects of ROIs. Buthionine sulfoximine (BSO) inhibits the rate-limiting enzyme in GSH synthesis, γ-glutamyl synthetase, and therefore depletes intracellular GSH. Pre-incubation of HeLa cells with BSO results in previously undetectable H_2O_2-induced NF-κB activation becoming apparent, although activation is low compared with TNF-α-stimulation (Li & Karin, 1999).

An alternative pathway of NF-κB activation has been reported in the presence of stimuli such as hypoxia, reoxygenation and the tyrosine phosphatase inhibitor pervanadate (Canty, Jr. *et al.*, 1999; Mukhopadhyay *et al.*, 2000). Here, it appears that instead of phosphorylation of serine residues, IκBα is phosphorylated on tyrosine 42; this leads to its removal without proteasomal degradation. Affinity purification to isolate proteins that specifically interact with tyrosine phosphorylated IκBα identified PI-3K as one such candidate. It appears that PI-3K recognises the phosphorylated tyrosine via by its src-homology 2 (SH2) domain and in this way sequesters the protein (Beraud *et al.*, 1999).

1.7 EXAMPLES OF PRO-INFLAMMATORY PROTEINS REGULATED BY NF-κB

1.7.1 Cyclo-oxygenase

Cyclo-oxygenase (COX) is a haemoprotein which is responsible for the conversion of arachidonic acid to prostaglandin H_2 (PGH_2) by its cyclooxygenase and peroxidase activities. Initially, activation of phospholipase A_2 liberates arachidonic acid from membrane phospholipids; this is converted to PGH_2 by COX, and then tissue-specific isomerases produce other prostaglandins, leukotrienes, thromboxanes and prostacyclin (Vane *et al.*, 1998).

There are two isoforms of this enzyme. COX-1 is a 71kDa, constitutively expressed protein which is found in the endoplasmic reticulum integrated into a single leaflet of the lipid bilayer. The lipid mediators produced via COX-1 are responsible for housekeeping functions including regulation of vascular homeostasis, gastric function, platelet reactivity and renal function. Along with many other cells types, monocytes and macrophages contain COX-1. The expression of COX-2 can be induced by specific stimuli (Kam & See, 2000). COX-2 is a 70kDa protein which shares 60% amino acid homology with COX-1. Like iNOS, COX-2 is a pro-inflammatory protein whose expression is up-regulated by NF-κB in response to inducers of inflammation and mitogens such as LPS, IL-1, TNF-α, epidermal growth factor-α (EGF-α) and IFN-γ.

The promoter of the COX-2 gene encodes binding sites for a number of transcription factors including NF-κB, NF-IL6 and cAMP-response element binding protein (CREB) although it appears that not all such elements are required for induction of COX-2 transcription. Transfection studies have been carried out in RAW 264.7 cells with a COX-2 promoter-luciferase reporter construct in order to investigate which transcription factors are required for LPS-stimulated promoter activity (Wadleigh *et al.*, 2000). The region –724 to +7 of the COX-2 promoter contains 2 binding sites for NF-IL6 as well as a binding site for NF-κB. In a series of experiments mutations of this promoter sequence were made to knock out each of the response elements. While

the NF-κB site is not essential for LPS-stimulated promoter activity, mutation of both NF-IL6 sites has a strong repressive effect. Thus, it seems that expression of the COX-2 gene does not require transcription factor binding at each site in the promoter. This redundancy was also illustrated in a study which used a similar transfection and reporter gene system to investigate transcription factor requirement for the human COX-2 gene. They found that the sequence from –327 to –52, which contains an NF-κB binding site, NF-IL6 binding site and cAMP response element (CRE), is necessary for LPS-induced luciferase activation. Mutation of individual sites showed that, while no single element is essential, a combination of at least 2 is required for activation of transcription. The presence of all 3 elements appears to have a synergistic effect on transcription, thus different pathways may activate different factors with several stimuli leading to maximum gene activation (Mestre *et al.*, 2000). Moreover, the importance of NF-κB in the expression of COX-2 has been shown in LPS-treated RAW 264.7 cells where inhibitors which block IκBα degradation or the nuclear translocation of NF-κB prevent the expression of COX-2 (Hwang *et al.*, 1997).

PGE_2 is the major prostaglandin produced in macrophages upon expression of COX-2 and this molecule exerts its effect by binding to the E-prostanoid (EP) receptors of which there are 4 subtypes (EP_{1-4}). EP receptors have 7 transmembrane domains and are G-protein coupled (Coleman *et al.*, 1994). Activation of these receptors can lead to the stimulation of adenylate cyclase and production of the second messenger adenosine-3'5'-monophophate (cAMP) or an increase in intracellular Ca^{2+}, depending on the particular subtype present (Coleman *et al.*, 1994).

1.7.2 Interleukin 6 (IL-6)

IL-6 is a 26kDa glycoprotein that has a variety of functions including immunoglobulin production, stimulation of T-cell proliferation, induction of hepatic acute phase proteins and stimulation of haemopoietic progenitor growth (Barton, 1996). IL-6 is a pro-inflammatory cytokine which can be up-regulated by NF-κB. The promoter for the IL-6 gene contains a consensus binding site for a number of transcription factors including NF-κB, CREB, AP-1 and NF-IL6. Transfection of IL-6

promoter/chloramphenicol acetyltransferase (CAT) reporter constructs into THP-1 cells indicates that <200bps upstream of the promoter gene is required for activation by LPS. This region contains two binding sites for NF-IL6 and an NF-κB binding site, with the NF-κB site appearing to be the strongest enhancer element (Zhang *et al.*, 1994).

IL-6 exerts its effects through binding to a receptor complex consisting of an IL-6 receptor (gp80) and a gp130 transmembrane protein (Chen-Kiang *et al.*, 1993). Binding of IL-6 causes association of the two receptor subunits, leading to activation of gp130 which is associated with members of the JAK family. JAKs then phosphorylate STAT3 on tyrosine residues which allows nuclear translocation and binding of this transcription factor to type I IL-6 response elements in promoter regions (Taga & Kishimoto, 1997). Stimulation of gp-130 by IL-6 bound to its receptor also leads to the activation of $p21^{ras}$ and phosphorylation and activation of the MAPK pathway. MAPK phophorylates NF-IL6 on threonine residues that results in its activation and binding to type II IL-6 response elements (Taga & Kishimoto, 1997).

1.8 REGULATION OF NOS EXPRESSION AND ACTIVITY BY NO

The activity of the constitutive NOS isoforms can be rapidly turned on and off by variations in intracellular calcium levels and phosphorylation/dephosphorylation. As the activity of iNOS is governed predominantly by protein expression, mechanisms which down-regulate activity must involve an effect on gene transcription, alter mRNA or protein stability or directly modulate enzyme activity. The unregulated production of NO by iNOS is deleterious for local tissues and is associated with the pathologies of a number of disorders including septic shock, rheumatoid arthritis and multiple sclerosis (McInnes *et al.*, 1996; Titheradge, 1999; Liu *et al.*, 2001). Therefore, mechanisms must exist by which the cell can rapidly turn off NO production from this 'high-output' NOS isoform.

1.8.1 Regulation of NOS activity by NO

NO itself can feed back to regulate the activity of NOS. This effect was initially reported to occur with constitutive NOS purified from rat cerebellum (Rogers & Ignarro, 1992). It has been shown that while oxyhaemoglobin (an NO scavenger) stimulates NOS activity, NO and NO donors have a concentration-dependent inhibitory effect. The effect of NO can be blocked by oxyhaemoglobin, suggesting that a reversible inactivation occurs, possibly through NO binding to the haem group of NOS. Subsequent studies have investigated the effect of NO on iNOS activity in LPS and IFN-γ-stimulated murine or rat macrophages. The activation of J774 murine macrophages in medium containing low levels of L-arginine or haemoglobin increases peak iNOS activity. Additionally, while NO donors inhibit iNOS activity, 8-bromo-cGMP has no effect suggesting a cGMP-independent mechanism (Assreuy *et al.*, 1993). A similar effect has been reported in rat alveolar NR8383 cells, with iNOS appearing much less sensitive to inhibition by NO than constitutive NOS (Griscavage *et al.*, 1993). In order to determine whether NO can inhibit activity through binding to the haem, the effect of other haem ligands on iNOS has been studied. Carbon monoxide, potassium cyanide and manganese-protoporphyrin IX all inhibit iNOS activity, although none of these compounds are as potent as NO (Griscavage *et al.*, 1993). Therefore it appears that NO can inhibit NOS activity by an interaction with the haem group. This represents a mechanism by which high levels of NO can rapidly block enzyme activity and prevent further NO production.

1.8.2 Regulation of iNOS expression by NO

Following the discovery that NO can modulate iNOS activity directly it became apparent that NO could also feed back to regulate expression of the enzyme. In astroglial cells derived from rat cerebral cortex where iNOS is induced with IL-1β and IFN-γ, iNOS activity is increased by the concomitant addition of the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), or oxyhaemoglobin with these compounds also leading to an increase in iNOS mRNA expression (Park *et al.*, 1994). A stimulation of iNOS mRNA expression in the presence of the NOS inhibitor, N^G-

methyl-L-arginine (L-NMA), has also been reported in LPS and IFN-γ-stimulated RAW 264.7 murine macrophage cells (Hinz *et al.*, 2000a). Furthermore, the inhibitory effect of NO on iNOS expression is also apparent in activated cells which are exposed to NO. In purified human microglia cells, pre-incubation with the NO donor sodium nitroprusside (SNP) leads to a decrease in LPS- and TNF-α-stimulated iNOS mRNA expression (Colasanti *et al.*, 1995). Therefore it seems that NO can feed back and inhibit the transcription of iNOS mRNA.

Despite the strong evidence in favour of a negative effect of NO on iNOS mRNA expression, certain studies suggest that NO may have a potentiating action on iNOS expression. Investigation of IL-1β and TNF-α-stimulated iNOS induction in human glomerular mesangial cells has shown that the NOS inhibitor L-NAME blocks iNOS expression while the NO donor SNP potentiates expression after 8 hours of activation (Perez-Sala *et al.*, 2001). Activation of the ANA-1 macrophage cell line with LPS and IFN-γ in the presence of increasing concentrations of the NO donor diethylamine NONOate (DEA-NO) demonstrates that, after 6 hours of stimulation, low concentrations of DEA-NO (1-12μM) lead to a 2.5-fold increase in iNOS mRNA expression while the highest concentration of DEA-NO (200μM) inhibits mRNA expression by 60%. A similar effect is also observed on iNOS protein expression and activity (Sheffler *et al.*, 1995). Thus the effect of NO on iNOS expression appears to be multi-faceted.

1.9 REGULATION OF NF-κB ACTIVITY BY NO

As NF-κB is the key transcription factor in iNOS expression, attention has focused on the modulation of this transcription factor by NO in an attempt to explain the above observations. In human peripheral blood mononuclear cells exposed to the NO donors SNP or S-nitroso-penicillamine (SNAP), the presence of NO leads to nuclear translocation and activation of NF-κB (Lander *et al.*, 1993). Treatment of RAW 264.7 cells with S-nitrosoglutathione (GSNO) results in a decrease in IκBα expression and activation of NF-κB. This effect is concentration-dependent, with higher concentrations of GSNO having no effect on activation (von Knethen *et al.*, 1999). A

similar effect is observed in rat striatal cultures where SNAP stimulation increases phosphorylation of IκBα and activates NF-κB (Simpson & Morris, 1999). In contrast to these studies where NO alone was found to activate NF-κB, it has also been found that in endothelial TC10 cells NF-κB activity is unaffected by treatment with the NO-releasing compound glycerol trinitrate (GTN) alone (Umansky *et al.*, 1998). However, when cells are stimulated with TNF-α, concentrations of GTN up to 250μM increase NF-κB activity while concentrations above this are inhibitory. Other studies have reported a negative effect of NO on NF-κB activity. In purified human microglia pre-treated with SNP a reduction in NF-κB activity and subsequent iNOS mRNA production is observed following LPS and TNFα activation (Colasanti *et al.*, 1995). An inhibitory effect of NO on NF-κB activity is also found in human saphenous vein endothelial cells activated with TNF-α in the presence of GSNO (Peng *et al.*, 1995). Additionally, it has been observed that GSNO is only effective as an inhibitor when added to whole cells rather than nuclear extracts, indicating the involvement of a cytosolic mechanism. Although a loss of IκBα is apparent in TNF-α treated cells, this is prevented by GSNO which also leads to an induction of IκBα. Therefore it appears that NO is blocking NF-κB activation by induction and stabilisation of IκBα. It has also been demonstrated that GSNO does not block phosphorylation or degradation of IκBα, but leads to a 2-3 fold stimulation of the re-synthesis of IκBα which is responsible for sequestering NF-κB and reducing its activity (Spiecker *et al.*, 1997).

Another mechanism by which NO appears to block NF-κB is via nitrosation of the p50 subunit. SNP leads to a concentration-dependent decrease in p50-DNA binding, an effect which is not observed with a $Cys^{62} \rightarrow$Ser p50 mutant (Cys^{62} is known to make close contacts with the κB binding motif). The study of a peptide which was composed of residues 43 to 77 of p50 by electrospray ionization mass spectrometry revealed that exposure to NO gas leads to an increase in mass of 29.1kDa which is indicative of nitrosation of the peptide (Matthews *et al.*, 1996). Thus it appears that NO can nitrosate the p50 subunit of NF-κB and directly block its DNA-binding activity. Subsequently it was shown that S-nitrosation leads to a 4-fold decrease in the equilibrium binding constant of recombinant p50 for its DNA target sequence (DelaTorre *et al.*, 1997). This effect has also been demonstrated *in vitro* using ANA-1

murine macrophage cells activated with LPS in the presence of S-nitroso-N-acetylcysteine (SNAC; DelaTorre *et al.*, 1999). Under these conditions there is no alteration in the nuclear levels of p50/p65, indicating that NO is acting on DNA binding activity rather than activation or nuclear translocation. Additionally, p50 protein isolated from LPS-treated cells has an absorption spectrum similar to S-NO-p50. When cells are activated in the presence of L-NAME this pattern is not apparent, indicating that nitrosation of p50 is a mechanism by which NF-κB is down regulated by endogenous NO.

An additional mechanism of inhibition of NF-κB activation by NO has been suggested by a recent study (Garban & Bonavida, 2001). Here, SNAP was shown to block TNF-α stimulation of NF-κB activity in human ovarian carcinoma AD10 cells, an effect that could be reversed by addition of H_2O_2. It has also been demonstrated that TNF-α stimulation of cells leads to an increase in H_2O_2 production and that this is blocked by incubation with the NO donor. It has been suggested that NO is exerting its inhibitory effect by interacting with O_2^- to form $ONOO^-$ and preventing the formation of H_2O_2 by SOD. Therefore NO is preventing the production of the ROI which potentiates NF-κB activation.

1.10 MECHANISMS OF NO MODULATION

1.10.1 cGMP-dependent effects

The mechanisms by which NO may produce opposing effects on NF-κB activity remain unclear. One possibility is that this effect is due partially to cGMP-dependent mechanisms. SNAP potentiation of COX-2 mRNA expression in IL-1β-activated rat primary mesangial cells is blocked by methylene blue (an inhibitor of sGC), and 8-bromo-cGMP (a cGMP analogue) increases COX-2 mRNA levels (Tetsuka *et al.*, 1996). In human glomerular mesangial cells 8-bromo-cGMP potentiates IL-1β and TNF-α activation of iNOS protein expression and nitrite production. It has also been found that the sGC inhibitor 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ)

reduces cytokine-stimulated iNOS protein expression by 41% (Perez-Sala *et al.*, 2001).

In contrast, a decrease NFκB activity and in iNOS expression and activity has been reported in murine bone marrow-derived macrophages activated with LPS in the presence of atrial natriuretic peptide (ANP) which activates particulate guanylate cyclase to increase cGMP levels (Kiemer & Vollmar, 1998). An inhibitory effect of ANP has also been reported in a similar experiment which looked at LPS-stimulated TNF-α production (Kiemer *et al.*, 2000). It was found that the cGMP analogue dibutyryl cGMP produced a similar concentration-dependent decrease in LPS-induced TNF-α production. ANP has also recently been demonstrated to dose-dependently inhibit LPS-induced L-arginine uptake in bone marrow-derived macrophages (Kiemer & Vollmar, 2001).

Other studies refute a role for cGMP in regulating NF-κB activity and pro-inflammatory protein expression. In certain studies, the cGMP analogue 8-bromo-cGMP has been found to have no effect on iNOS mRNA levels or nitrite production in LPS and IFN-γ-activated RAW 264.7 macrophages (Hinz *et al.*, 2000a). Also, in contrast to the results reported above, methylene blue, has no effect on COX-2 expression in LPS-stimulated RAW 264.7 cells (Salvemini *et al.*, 1993). Therefore it remains to be confirmed whether cGMP is involved in the regulation of pro-inflammatory protein expression by NO, and whether cGMP can influence NF-κB activity.

1.10.2 Direct effects of NO (cGMP-independent)

NO may also modify NF-κB activity and pro-inflammatory protein expression by directly modifying upstream signalling molecules in the activation pathway. One molecule which has been identified as having a role in LPS-induced NF-κB activation is $p21^{ras}$ (Pahan *et al.*, 2000) and this is also a molecule which appears to be sensitive to modulation by NO. This was demonstrated by looking at the influence of NO on the activation of $p21^{ras}$ in the Jurkat human T cell line. Lower concentrations of NO

(1-10nM) enhance the exchange of GDP for GTP on $p21^{ras}$ which leads to activation of the molecule. This effect is reversed by the addition of haemoglobin, indicating that NO can bind to and reversibly modify the protein (Lander *et al.*, 1995a). A subsequent study revealed that Cys^{118} of $p21^{ras}$ is a target for S-nitrosation with NO-stimulated GDP to GTP exchange being blocked when this residue is mutated to Ser (Lander *et al.*, 1995b). It appears that activation of $p21^{ras}$ by NO can lead to the recruitment and activation of PI-3K (Deora *et al.*, 1998) and Raf-1 (Deora *et al.*, 2000) which may result in increased NF-κB activation and iNOS/COX-2 expression.

1.11 NOS AND COX INTERACTIONS

There are similarities between the NOS and COX enzyme systems in that they both have constitutive and inducible isoforms, with the inducible isoforms being up-regulated by inflammatory stimuli. Indeed, both promoter regions of iNOS and COX-2 have consensus binding sites for NF-κB, AP-1 and NF-IL6 (see Chapters 1.2.4 and 1.7.1). As the activities of NOS and COX are both up-regulated under similar conditions many investigations have focused on whether the mediators produced (eg. NO, PGE_2) interact to regulate the sibling system. Since NO has been shown to have an effect on NF-κB activity it may also modulate COX-2 expression. Additionally, the COX enzymes have an iron-haem centre at their active site which may be susceptible to modification by NO, as is NOS.

In LPS-stimulated RAW 264.7 the NOS inhibitors L-NAME and aminoguanidine (AG; iNOS specific) both lead to a concentration-dependent decrease in nitrite and also PGE_2 production. The inhibition of NOS activity by activation of cells in L-arginine free medium also leads to a four-fold decrease in PGE_2. In contrast, the COX inhibitor indomethacin blocks PGE_2 production but has no effect on nitrite levels (Salvemini *et al.*, 1993). These findings have also been repeated *in vivo*, with NOS inhibitors decreasing the levels of prostaglandins in the plasma and urine of LPS-treated rats while COX inhibitors have no effect on levels of nitrite secreted (Salvemini *et al.*, 1995). The effect of NO on COX has been confirmed by the finding that SNP stimulates PGE_2 production by recombinant COX-1 or 2, which suggests a direct effect on enzyme activity (Salvemini *et al.*, 1993).

Subsequent studies have also investigated whether the effect of NO on COX activity occurs via a change in enzyme expression. Activation of rat primary mesangial cells with IL-1β and increasing concentrations of the NO donors SNAP or SNP leads to an increase in COX-2 mRNA, protein and activity. This effect is only apparent in conjunction with IL-1β, as NO donor alone does not induce COX-2 expression or activity (Tetsuka *et al.*, 1996). A positive effect of NO on COX activity has also been reported in cytokine-stimulated human astrocytoma cells (Mollace *et al.*, 1998), with L-NAME inhibiting IL-1β and TNF-α-stimulated PGE_2 production and SNP having a potentiating effect. Stimulation of COX-2 protein expression by GSNO, both alone and in conjunction with LPS, has also been reported (von Knethen *et al.*, 1999; von Knethen & Brune, 2000).

A potentiating effect of NO on COX-2 activity has been suggested by studies carried out in iNOS KO mice. In a rodent model of focal cerebral ischaemia the ischaemia-associated increase in PGE_2 was attenuated by 40-50% in iNOS KO animals. This appears to be due to a deficiency in NO-stimulated COX-2 activity as expression levels were unaffected (Nogawa *et al.*, 1998). In peritoneal macrophages isolated from iNOS KO mice, a decrease in LPS and IFN-γ-stimulated PGE_2 production, as compared with WT macrophages is observed; again there is no corresponding change in COX-2 protein levels (Marnett *et al.*, 2000).

While in the above models it appears that NO potentiates PGE_2 production, in cartilage taken from human osteoarthritis patients NO has the opposite effect (Amin *et al.*, 1997). In this tissue, administration of the NOS inhibitor L-NMA leads to a 2-fold augmentation of PGE_2 levels while SNP is inhibitory. An inhibitory effect of NO on COX-2 activity and expression in RAW 264.7 cells has also been reported (Patel *et al.*, 1999). Furthermore, endogenous and exogenous NO inhibit both COX-2 expression and activity in LPS-treated J774.2 murine macrophages although in the absence of endogenous NO, exogenous NO stimulates COX-2 activity (Swierkosz *et al.*, 1995).

The effects of selective inhibitors of NOS and COX isoforms on plasma levels of 6-keto-$PGF_{1\alpha}$ (the stable metabolite of prostacyclin) and nitrite/nitrate (NO_x) have been

investigated in LPS-treated rats. The iNOS inhibitor, [N-(3-aminomethyl)benzylacetamine, 2HCl] (1400W), and the non-selective NOS inhibitor, L-NMA, have no effect on 6-keto-$PGF_{1\alpha}$ while the COX-2 inhibitor celecoxib and the non-selective COX inhibitors diclofenac or A771726 do not modulate NO_x (Hamilton & Warner, 1998). This suggests there is not an interaction mediated by the products of these enzymes.

The different effects of NO on COX expression and activity may be due to the use of different concentrations of NO-donors and NOS inhibitors, or variations in the particular time when expression is measured. Both positive and negative effects of NO on COX-2 mRNA and protein expression have been reported in human mesangial cells depending on the time of intervention (Diaz-Cazorla *et al.*, 1999). It is also possible that NO may have different effects, depending on whether it is acting on the COX-1 or COX-2 isoform. In $COX\text{-}1^{-/-}$ or $COX\text{-}2^{-/-}$ murine lung fibroblasts treated with NO, 1μM NO stimulates PGE_2 production in $COX\text{-}2^{-/-}$ cells but has no stimulatory effect on $COX\text{-}1^{-/-}$ fibroblasts, suggesting NO is activating only COX-1 (Clancy *et al.*, 2000).

The effects of PGE_2 and cAMP (which is produced in response to PGE_2) on iNOS activity and expression have also been investigated and again conflicting results have been obtained. Differing effects of cAMP-raising agents have been found on LPS-stimulated cell types. While forskolin, which activates adenylate cyclase, and cAMP analogues decrease iNOS expression and activity in rat primary astrocytes, they have the opposite effect in rat peritoneal macrophages (Pahan *et al.*, 1997). Further evidence for a cell type-specific effect of cAMP on iNOS is indicated by the inhibition of LPS and cytokine-stimulated nitrite production in rat hepatocytes but the potentiation of nitrite production in rat pulmonary artery smooth muscle cells (Harbrecht *et al.*, 2001). Several studies have reported that PGE_2 and cAMP inhibit iNOS expression and activity in J774.1 macrophages (Pang & Hoult, 1997; D'Acquisto *et al.*, 1998) and rat Kupffer cells (Mustafa & Olson, 1998). This effect may occur via modulation of NF-κB activity by PGE_2. Human peripheral blood monocytes incubated with LPS and fibroblast-conditioned medium show a decrease in NF-κB activity as compared with activation with LPS alone. This effect is blocked

when the fibroblasts are incubated with the COX inhibitor indomethacin, indicating that inhibition of NF-κB activity is occurring due to the presence of products of COX. Indeed a concentration-dependent inhibition of NF-κB activity is apparent when the cells are incubated with LPS and increasing concentrations of PGE_2 (Conte *et al.*, 1997). A decrease in LPS and cytokine-stimulated NF-κB activity is also observed in rat hepatocytes exposed to dibutyryl-cAMP (Harbrecht *et al.*, 2001). However, in a more recent study, where inflammatory mediators have been monitored in a mouse model of trauma, the COX-2 inhibitor NS-398 leads to a decrease in levels of TNF-α, IL-6, NO and COX-2 mRNA (Mack *et al.*, 2001). Furthermore it has also been found in RAW 264.7 macrophages that cAMP-elevating agents stimulate LPS-induced iNOS expression while agents which block cAMP-dependent protein kinase (A-kinase) are inhibitory (Chen *et al.*, 1999). Thus, it appears in these studies that COX-2 products are increasing NF-κB activity.

1.12 CONSTITUTIVE NOS AND THE MACROPHAGE

Although iNOS is generally accepted as the isoform of NOS which is expressed predominantly in macrophages there are also reports of expression of constitutive NOS in these cells. NOS activity has been detected in membrane extracts of non-induced J744.1 macrophages which appears to be dependent on the presence of Ca^{2+} (Hecker *et al.*, 1992). In peripheral blood mononuclear cells, a band can be detected by reverse transcriptase polymerase chain reaction (RT-PCR) using primers designed from human eNOS (Reiling *et al.*, 1994) and this is also apparent in the human monocytic U937 cell line (Roman *et al.*, 1997). In U937 cells eNOS can be detected by flow cytometry using eNOS-specific antibodies and the activity of this protein is Ca^{2+}-dependent and requires the presence of calmodulin (Roman *et al.*, 1997). In freshly isolated rat alveolar macrophages a low level of NO production can be detected and western blotting of cell extracts reveals a band with an anti-eNOS antibody but not with antibodies directed against iNOS or nNOS. Although the activity of this enzyme is stimulated by the addition of Ca^{2+}, it differs from eNOS in that removal of Ca^{2+} and Mg^{2+} does not affect activity. A lower level of NO is produced than would be expected from eNOS and activity is stimulated by external L-arginine, which is a characteristic more associated with the inducible isoform (Miles

et al., 1998). Therefore, while these reports suggest the existence of a constitutive NOS isoform in macrophages it is unclear if this is in fact eNOS or a novel isoform. Moreover, any role for this isozyme in macrophage activity has yet to be demonstrated.

1.13 CURRENT STUDY

In the current study the RAW 264.7 murine macrophage cell line and murine bone marrow-derived macrophages have been used to investigate the effect of NO on NF-κB activity and the expression of pro-inflammatory proteins.

1. The effects of exogenous and endogenous NO on LPS-stimulated macrophage activation have been investigated in order to help clarify previous findings which attribute pro- and anti-inflammatory actions to NO.

2. Bone marrow-derived macrophages have been cultured from eNOS and iNOS KO mice in order to determine which NOS isoforms are responsible for the effects of NO on iNOS and COX-2 expression and activity.

3. The involvement of cGMP-dependent mechanisms has been investigated by observing the effects of sGC activating and inhibitory compounds on LPS-stimulated protein expression.

4. The existence of cross-talk between NOS and COX isoforms has been studied by the use of KO animals and compounds which modulate cGMP or cAMP levels and COX activity.

CHAPTER TWO
MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 REAGENTS

Salmonella typhosa (*S. typhosa*) Lipopolysaccharide (LPS; serotype 0901) was obtained from Difco Laboratories (Detroit, USA), resuspended in distilled water to a concentration of 10mg/ml, aliquoted and stored at –20°C. LPS was diluted in experimental medium before addition to cells. Diethylamine-NONOate (DEA-NO) was obtained from Caymen Chemicals (supplied by Alexis, Nottingham, UK) and resuspended in experimental medium immediately before addition to cells. N^G-nitro-L-arginine methyl ester (L-NAME) was purchased from Sigma (Poole, UK) and diluted in experimental medium prior to experiments. Forskolin, N-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide (NS-398) and 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole (SC-560) were purchased from Calbiochem (Nottingham, UK). BAY 41-2272 was a gift from Bayer AG, Germany. 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) was purchased from Sigma. Each of these drugs were resuspended in dimethyl sulphoxide (DMSO) and diluted further in medium (NS-398 and SC-560) or added directly to cells (forskolin, ODQ and BAY 41-2272; final concentration of DMSO did not exceed 0.001%). [N-(3-aminomethyl)benzylacetamine, 2HCl] (1400W) was a gift from Glaxo Smith Kline, UK and was diluted in medium before experiments. All other reagents were purchased from Sigma.

2.2 RAW 264.7 CELL CULTURE

RAW 264.7 cells (TIB 71; American Type Culture Collection, Manassas, USA) were cultured in RPMI 1640 medium (with 25mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; HEPES) supplemented with 10% heat-inactivated New Zealand foetal bovine serum (FBS; low endotoxin: 0.019ng/ml, 0.236 endotoxin units/ml), 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin (all Life Technologies, Paisley, UK; complete medium). The cell cultures were maintained in a glass stirrer bottle at 37°C in a humidified incubator containing 5% CO_2 in air. Cell

viability and number were measured by trypan blue exclusion. Equal volumes of cell suspension and Trypan Blue (Life Technologies) were mixed and placed on a haemocytometer; cells were counted under a microscope and luminescent cells were counted as live and blue cells were counted as dead.

In L-arginine-free experiments medium was prepared using RPMI 1640 (with 25mM HEPES) free from L-arginine (Life Technologies) supplemented with 10%, dialyzed heat inactivated New Zealand FBS (low endotoxin), and glutamine, penicillin and streptomycin as described above (L-arginine-free medium). FBS was dialyzed for 3 x 24 hrs in Dulbecco's Phosphate Buffered Saline (PBS; without calcium chloride and magnesium chloride; Life Technologies) at 4°C.

2.3 EXPERIMENTATION WITH RAW 264.7 CELLS

2.3.1 NOS inhibition/NO donor studies

RAW 264.7 macrophages were incubated for 24 hrs in 75-cm^2 flasks at a concentration of 5 x 10^6 cells/flask in 10ml of complete medium. Before activation with LPS (1μg/ml), the medium was removed, and the cells were washed with 5ml PBS and replenished with complete medium or L-arginine free medium. In certain experiments DEA-NO (30nM-300μM) was added concomitantly with LPS or cells were pre-incubated for 15 mins with L-NAME (1mM) or 1400W (10μM) before activation with LPS. Flasks were then incubated as previously. At appropriate time points, medium was removed from flasks, and an aliquot was stored at –20°C for IL-6, PGE_2 and nitrite measurement. The remaining medium was discarded, cells were washed with 5ml of PBS and following addition of 1.5ml Accutase (TCS, Botolph Claydon, UK), flasks were incubated for an additional 10 mins. Cells were dislodged and washed three times by centrifugation (200 x g, 5mins, 4°C) with PBS, and pellets were stored at –80°C. Viability was measured by trypan blue exclusion.

2.3.2 COX inhibitor studies

RAW 264.7 cells were incubated for 24 hrs in 6-well plates at a concentration of 1 x 10^6 cells/well in 2ml of complete medium. Before activation with LPS (1μg/ml), the medium was removed and each well was washed with 0.5ml PBS and replenished with complete medium. The cells were then pre-incubated for 30 mins with SC-560 (300nM) or NS-398 (30μM), before activation with LPS. At appropriate time points medium was removed and an aliquot was stored at –20°C for PGE_2 and nitrite measurement. The remaining medium was discarded and the cells washed with 0.5ml PBS before addition of 100μl whole cell homogenisation buffer (50mM Tris[hydroxymethyl]aminomethane (Tris HCl), 150mM NaCl, 1%Triton X-100, 2mM ethylenediaminetetraacetic acid (EDTA), 8mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10μg/ml protease inhibitor cocktail (benzamidine, antipain, leupeptin and aprotinin)) per well. Plates were incubated on ice for 20 mins after which each well was scraped and the homogenate transferred to a 1.5ml tube. The wells were washed with a further 100μl of buffer to give a total of 200μl of homogenate which was incubated on ice for a further 20 mins. A whole cell extract was made in order that COX-1 expression could be followed as this protein is membrane-associated. The tubes were then centrifuged (13,793 x g, 5mins, 4°C), the supernatants retained and stored at –80°C.

2.4 BONE MARROW-DERIVED MACROPHAGES: EXTRACTION AND CULTURE

2.4.1 Preparation of L929 supernatant

The supernatant from L929 fibroblasts was used as a source of M-CSF for the conditioning medium. L929 fibroblasts (clone 6; gift from Dr Manuel Modollel, Max Planck Institute for Immunobiology, Freiburg, Germany) were cultured in 175-cm^2 flasks in Dulbecco's Modified Eagles Medium (DMEM; high glucose; 4.5g/L) with L-glutamine and sodium pyruvate and supplemented with 10% heat-inactivated New Zealand FBS (as above), 100U/ml penicillin, 100μg/ml streptomycin, 50μM 2-mercaptoethanol and 1% 100X MEM essential amino acids without L-glutamine (all

Life Technologies) at 37°C in a humidified incubator containing 10% CO_2 in air. Cells were passaged by trypsinisation then seeded to confluence and after 5-7 days of culture the supernatant (growth medium) was collected, centrifuged (593 x g, 10mins) then filtered through a 0.2μm membrane, aliquoted and stored at –80°C.

2.4.2 Extraction and culture of bone marrow-derived macrophages

eNOS KO mice were generated from the C57B16 strain of mouse through the disruption of the eNOS gene with a neomycin insert which replaced amino acids 1010-1144 (Huang *et al.*, 1995). iNOS KO mice were generated from C57BL/6J mice also by a neomycin gene insertion replacing exons 12 and 13 of the iNOS gene (Laubach *et al.*, 1995). The mice used in the current study were bred at the Wolfson Institute from breeding pairs that were generated as above. Femurs were removed from 6-8 weeks old, male WT and KO mice and placed in collection medium (DMEM with HEPES and without $NaHCO_3$ supplemented with 10% heat-inactivated New Zealand FCS (low endotoxin), and 100U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol). The ends of each bone were removed using scissors and bone marrow cells were flushed out using a 27G needle and a syringe filled with collection medium. Cells were resuspended by pipetting, centrifuged (461 x g, 10mins) and resuspended in conditioning medium (DMEM; high glucose; 4.5g/L with L-glutamine and sodium pyruvate and supplemented with 10% heat-inactivated New Zealand FBS (low endotoxin), 100U/ml penicillin, 100μg/ml streptomycin, 10% L929 supernatant, 5% heat-inactivated horse serum, 1% 100X MEM essential amino acids without L-glutamine and 50μM 2-mercaptoethanol). Cells were counted using trypan blue exclusion and between 3 and 6 x 10^6 cells were seeded in a total of 50ml conditioning medium per teflon bag (approximately 4 bags of cells were generated per animal). The teflon bags were formed by heat-sealing around 3 edges of a piece of teflon fluorocarbon film (Dupont, Hemel Hempstead, UK), such that the cells grew on the hydrophobic side. The resulting bags (5 x 30cm) were packaged and sterilised using ethylene oxide. After filling, the open end of each bag was heat sealed and bags laid on metal trays with circular holes on the bottom (to allow gas exchange) which were incubated at 37°C in a humidified incubator containing 10% CO_2 in air. After 10 days bags were removed and massaged to ensure the cells were in suspension. The

culture was then removed using a syringe fitted with a 14G I.V catheter (Johnson and Johnson, Ascot, UK) and transferred to a 50ml centrifuge tube. Cells were pelleted by centrifugation (259 x g, 10mins) and resuspended in complete medium (as previously). Cells were then seeded into 12-well culture plates at a concentration of 1 x 10^6 cells/well in 1ml complete medium and incubated overnight at 37°C in a humidified incubator containing 5% CO_2 in air. Cells were then activated with LPS (100ng/ml) with 2 wells used for each condition. In certain experiments cells were pre-incubated for 30mins in the presence of increasing concentrations of BAY 41-2272 (0.1-10μM), ODQ (10μM) or forskolin (0.1-50μM) prior to addition of LPS (100ng/ml). At appropriate time points, 0.5ml medium was taken from each of the duplicate wells and the combined 1ml was stored at –20°C for PGE_2 and nitrite measurement. The remaining medium was discarded and wells washed with 0.5ml PBS. 50μl whole cell homogenisation buffer (as previously) was added per well and plates incubated on ice for 20mins. The two wells were then scraped and homogenates combined in a 1.5ml tube, a further 50μl was used to wash both the wells and added for a total of 150μl of homogenate. After 20mins on ice, tubes were centrifuged (13,793 x g, 5mins, 4°C) and supernatants retained for Western Blotting.

2.4.3 Characterisation of bone marrow-derived cells by flow cytometry

After cells were harvested from teflon bags, the cell populations obtained were characterised. Expression of the cell surface marker F4/80, which is specific for murine macrophages, and the lymphocyte-specific CD3, were detected by flow cytometry. This method of analysis separates cells on the basis of size as well as providing a quantitative assessment of cell surface immunoflourescence which can be determined by the binding of flourescent-labelled antibodies. Four aliquots of 2 x 10^5 cells from WT or KO animals were incubated with flourescein isothiocyanate (FITC) conjugated antibodies (all supplied by Serotec, Oxford, UK) raised against F4/80 and CD3, and isotype control antibodies (rat IgG2b negative control and rat IgG2a negative control), which had been diluted to a concentration of 300-500ng/ml in 200μl PBS containing 1% heat-inactivated New Zealand FBS (both Life Technologies). After 2 hrs incubation at room temperature cells were washed twice by centrifugation (200 x g, 2 mins) with PBS/1%FBS. Cells were then resuspended in

200µl fixation solution (1% paraformaldehyde in PBS) and incubated at room temperature for 15 mins. The cells were centrifuged as before and resuspended in 200µl PBS/1%FBS. The flow cytometry was performed using a FACScalibur machine (Becton Dickinson, Oxford, UK). The cell suspension forms a liquid jet which passes through a laser beam and the pattern of light scatter created then passes through detectors which process the scatter pattern. The light scatter pattern is determined by the size and internal structures of the cells and the immunoflourescence of the cell surface can also be measured. The FACS machine allows statistical analysis of the data and cells can be characterised according to the above parameters. The photomultiplier detectors (PMT) which measure light scatter were set to an optimal level for the bone marrow-derived cell population. These were Forward Scatter Channel (FSC) – E-01, Side Scatter Channel (SSC) – 400, FL-1 channel – 500. The fluorescence of each marker and control was determined in the FL-1 channel. The statistical analysis was performed using Cell Quest software. Dot plot analysis was used to analyse the size and granularity of the cells. The use of gating allowed statistical analysis to be perfomed on the live cells separate from the debris. Histogram analysis allowed the separation and quantitation of the cell population according to flourescence of the marker.

2.5 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND WESTERN BLOTTING

2.5.1 NOS inhibition/NO donor studies - protein preparation

Cells were homogenized in 50mM TrisHCl, pH 7.5, 0.1mM dithiothreitol (DTT), 10µg/ml protease inhibitor mix (benzamidine, leupeptin, aprotinin, antipain) by sonication. The resulting homogenate was centrifuged (105,000 x g, 30mins, 4°C) and the supernatant retained (cytosolic extract). Protein concentrations were determined using the Biorad Protein Assay Kit II (Bio-rad, Hemel Hempstead, UK). This assay is based on the method of Bradford and involves the use of a Coomassie blue dye which binds to basic and aromatic amino acid residues. Such binding reactions lead to a shift in the absorbance maximum for the dye from 465 to 595nm. A standard curve was constructed by adding the dye to bovine serum albumin which had been diluted (0.25-

1mg/ml) in homogenisation buffer (Figure 4). Samples were diluted in homogenisation buffer in order that they gave a reading which fell on the standard curve. The absorbance at 595nm was measured for standards and samples using a 96-well microplate reader (Molecular Devices, Wokingham, UK). Samples were then diluted in their homogenisation buffer so that the concentration of each was equivalent to the sample with the lowest concentration.

2.5.2 COX inhibitors and bone marrow-derived macrophage studies – protein assay

Protein concentrations were measured using the BCA Protein Assay Kit (Pierce, Rockford, USA), as whole cell homogenisation buffer was incompatible with the Biorad Protein Assay Kit II. The BCA kit works by the reduction of copper ions present in the BCA reagent by proteins in the samples. The reduced copper then reacts with Bicinchoninic Acid in the reagent to give a coloured complex of which the absorbance can be measured at 562nm. A standard curve was prepared using bovine serum albumin diluted between 0.125-2mg/ml (Figure 4). The absorbance of standards or sample mixed with BCA reagent was measured using a plate reader as above. Samples were diluted as above in whole cell homogenisation buffer. In experiments with higher protein concentrations each sample was diluted to 1mg/ml.

STANDARD CURVES FOR BIORAD AND BCA PROTEIN ASSAY KITS

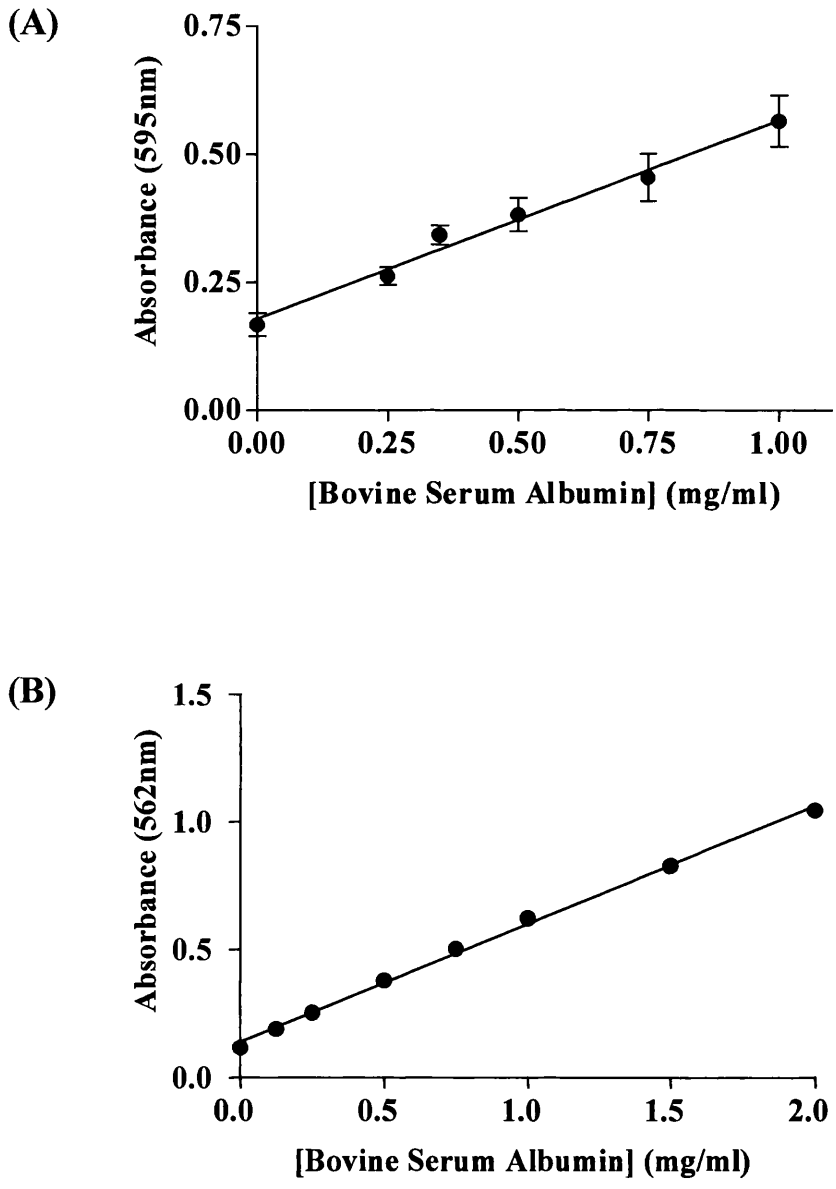


FIGURE 4. Standard curves for (A) Biorad Protein Assay kit and (B) BCA Protein Assay kit prepared with increasing concentrations of bovine serum albumin. Protein was diluted in homogenisation buffer and assay reagent added before measurement of absorbance by 96-well plate reader. Data are represented as mean ± SEM absorbance for each concentration (n >3).

2.5.3 SDS-PAGE

SDS-PAGE was carried out using the Mini Protean II or III Gel tanks (Bio-rad). The separating gel was poured containing 7.5% acrylamide (acrylamide/bis-acrylamide 30% solution, mix ratio 37.5:1 purchased from Sigma), separating gel buffer (0.375M Tris Base, 0.1% SDS in distilled H_2O, pH 8.8), 0.1% ammonium persulphate and 0.001% TEMED in distilled H_2O. A layer of isopropanol was placed on top of the gel and removed after the gel had set. The stacking gel was poured containing 4% acrylamide, stacking gel buffer (0.125M Tris Base, 0.1% SDS in distilled H_2O), 0.1% ammonium persulphate and 0.001% TEMED in distilled H_2O. The combs were inserted and gels allowed to set. Combs were removed and wells washed with distilled H_2O before assembly of the apparatus and addition of running buffer (50mM Tris Base, 0.384M glycine and 0.1% SDS in distilled H_2O). A 1:1 dilution was made for each homogenate in 2 x sample buffer (20mM Tris HCl, pH 6.8, 2mM EDTA, 2% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.025% bromophenol blue in distilled H_2O) and samples were heated at 95°C for 4 mins in a heating block (Grant, Cambridge, UK) immediately before loading on the gel. 25µl of sample was loaded per lane and these were run alongside 10µl of high molecular weight rainbow marker (Amersham Pharmacia, Little Chalfont, UK). Gels were run at 80-120V.

2.5.4 Western Blotting

A sandwich was made with filter paper (Munktell Filter Paper, Grade 1F, Amersham Pharmacia), nitrocellulose (Hybond ECL, Amersham Pharmacia) and the SDS gels. The following transfer sandwich was set up on a Multiphor II semi-dry blotter (Amersham Pharmacia): 6 sheets of filter paper soaked in Solution 1 (0.3M Tris Base (pH 10.4), 20% methanol in distilled H_2O), 3 sheets of filter paper soaked in Solution 2 (0.025M Tris Base (pH 10.4), 20% methanol in distilled H_2O), nitrocellulose soaked in Solution 2, SDS gel and 9 sheets of filter paper soaked in Solution 3 (0.04M 6-amino-n-hexanoic acid (pH 7.6), 20% methanol in distilled H_2O). Proteins were transferred at 0.8mA/cm^2 for 60mins. The membranes were then incubated, shaking in 5% milk in wash buffer (3.25mM NaH_2PO_4, 7.5mM Na_2HPO_4, 0.145M NaCl and 0.001% Tween in distilled H_2O) for 1 hr at room temperature. The membrane was

washed twice (15mins/wash with shaking) in wash buffer before incubation overnight, at 4°C with gentle shaking, with primary antibody (αiNOS: Transduction Laboratories (Becton Dickinson); αCOX-1 and αCOX-2: Cayman Chemicals (supplied by Alexis); αactin: Chemicon, Harrow, UK; αeNOS: Santa Cruz (supplied by Autogen Bioclear, Calne, UK) diluted 1:2000 (αiNOS, αeNOS), 1:3000 (αCOX-2), 1:1000 (αCOX-1) or 1:10,000 (αactin) in 1% milk in wash buffer. The membrane was washed 6 times (5 mins/wash with shaking) and then incubated, with gentle shaking for 2 hrs at room temperature, with horse radish peroxidase (HRP)-conjugated αrabbit IgG (Vector, Peterborough, UK) or HRP-conjugated αmouse IgG (Dako, Glostrup, Denmark) diluted 1:5000 (αrabbit) or 1:2000 (αmouse) in 1% milk in wash buffer. The membrane was washed as previously and proteins were visualised using enhanced chemiluminescence (ECL Detection Kit, Amersham Pharmacia) according to the manufacturer's instructions. Membranes were then exposed to Kodak X-OMAT AR-5 film (supplied by BDH Laboratory Supplies, Poole, UK) and developed using an automatic developer (Compact X4, X-ograph, Wiltshire, UK). The resulting images were scanned and bands were quantified by densitometry (NIH Image).

2.6 RNA ISOLATION AND REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

RNA was extracted from cells using a SNAP Total RNA Isolation Kit (Invitrogen, Leek, The Netherlands). Cell pellets were resupended in lysis buffer and the samples passed three times through a syringe fitted with a 19G needle. Isopropanol was added and samples mixed before being centrifuged through a nucleic acid extraction column. The columns were washed and the nucleic acid was eluted in RNase-free water. The sample was then incubated with DNase to destroy DNA present and the column binding, washing and elution steps were repeated. Random hexamers (0.15ng/reaction; Life Technologies) were added to equal amounts of RNA made up to 12μl volume in diethyl pyrocarbonate-treated water. The mixture was heated at 70°C for 10mins, then cooled immediately on ice. DTT (10mM), first strand buffer (supplied with enzyme) and PCR nucleotide mix (0.5mM; Roche Molecular Biochemicals, Lewes, UK) were added to a final volume of 20μl and the mix was

heated at 25°C for 10mins. Reverse transcription was then carried out at 42°C for 55mins with 200U Superscript II RNase H reverse transcriptase (Life Technologies) added after 2mins of incubation. The reaction was then heated at 70°C for 15mins and stored at –20°C.

PCR was performed on a Primus 96 Thermocycler (MWG Biotech, Milton Keynes, UK) in a reaction containing 0.2mM PCR nucleotide mix (Roche Molecular Biochemicals), forward and reverse primers (1μM each), 1 x PCR reaction buffer with $MgCl_2$ (supplied with enzyme), 1μl cDNA and 1 unit *Taq* DNA polymerase (Roche Molecular Biochemicals) made up to 25μl with distilled water. The same master mix containing all reagents was used for each sample. Preliminary experiments were performed to ensure that the number of cycles used gave a product quantity which was on the linear portion of the PCR amplification curve (Figure 5). Thermal cycling conditions were as follows: 95°C for 5mins then 22 cycles of denaturation at 95°C for 1min, annealing at 58°C for 2mins and polymerisation at 72°C for 2mins followed by a final extension at 72°C for 10mins.

Primer sequences (Sigma-Genosys, Cambridge, UK):

iNOS sense	5'-GCATTTGGGAATGGAGACTG-3'
iNOS antisense	5'-GTTGCATTGGAAGTGAAGCGTTTC-3'
COX-2 sense	5'-GAGGTACCGCAAACGCTT-3'
COX-2 antisense	5'-TTATTGCAGATGAGAGACTG-3'
GAPDH sense	5'-ATGGTGAAGGTCGGTGTGAACG-3'
GAPDH antisense	5'-GGCGGAGATGATGACCCGTTTGGC-3'

PCR products were resolved by agarose gel electrophoresis on a 2% gel. Electrophoresis Grade Agarose (Life Technologies) was dissolved by heating in 0.04M Tris-acetate, 0.001M EDTA in distilled H_2O (TAE) with 0.375μg/ml ethidium bromide (aqueous Solution, Sigma) added before the gel set. 2μl of 6 x Gel loading solution (Sigma) was added to each reaction and the entire volume was loaded onto the gel. 5μl of 100 base pair DNA ladder (Life Technologies) was run alongside samples. Gels were run between 100-200V in TAE and then visualised and photographed using a Multi Image Light Cabinet and Chemi Imager 4400 v5.1

software (Alpha Innotech Corporation, supplied by Flowgen, Ashby de la Zouche, UK).

PCR AMPLIFICATION CURVE

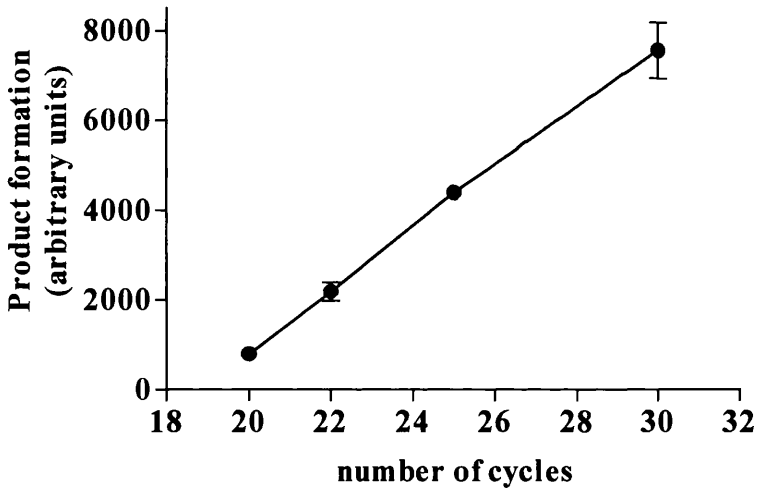


FIGURE 5. Intensity of iNOS PCR bands in RAW 264.7 cell extracts activated with LPS (1μg/ml). PCR reactions were carried out using increasing numbers of polymerisation cycles and bands were analysed by densitometry. Data are represented as the mean ± SEM density under each condition expressed in arbitrary units (n > 3).

2.7 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

RAW 264.7 cells (7 x 10^6) were seeded in 10ml complete medium in 10cm tissue culture dishes. Cells were incubated as previously overnight then medium was aspirated and cells were washed with 2ml PBS and medium replaced before activation with 1μg/ml LPS. After appropriate time points medium was removed and nuclear extracts were prepared by washing adherent cells with cold PBS (2 x 1ml) and adding cold buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 % Nonidet P-40). The dish was scraped and the reaction volume centrifuged (13,397 x g, 1min, 4°C). Supernatants were discarded, and the nuclear pellets were resuspended in 60 μl of buffer C (20 mM HEPES pH7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM EDTA) and left on ice for 10min. To both A and C buffers, a protease inhibitor cocktail tablet (Roche Molecular Biochemicals) was added. The solution was centrifuged (13,397 x g, 2mins, 4°C) and the supernatants were recovered as nuclear extracts. The protein concentrations were measured using the Biorad Protein Assay II Kit. Samples were aliquoted and stored at –70°C. A double-stranded oligonucleotide containing an NF-κB consensus sequence (5'-GGGGACTTTCC-3'; Promega, Southampton, UK) was end-labelled using [γ-32P]-ATP and T4 polynucleotide kinase (Promega). Probes were purified using Centri-sep Spin columns (Princeton separations, Sigma-Genosys). For each experiment protein-DNA binding reactions were performed using equal amounts of nuclear extract protein (2-3 μg) and labelled oligonucleotide in the presence of incubation buffer (1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH7.5), 0.05 μg/μl poly dIdC, 4% glycerol) for 30 mins on ice. To ensure specificity of probe binding, certain experiments were conducted in the presence of 100-fold molar excess of unlabeled (cold) NF-κB consensus oligonucleotide (Figure 6). Protein-DNA complexes were resolved on 5% polyacrylamide gels, electrophoresed for 1 hr at room temperature in 0.5 x TBE (45 mM Tris-borate, 1 mM EDTA pH 8.0). Gels were exposed to X-ray film (Kodak X-OMAT AR-5) overnight at –80°C before developing using a Compact X4 developer (X-ograph). The resulting images were scanned and bands were quantified by densitometry (NIH Image).

SPECIFICITY OF NF-κB PROBE

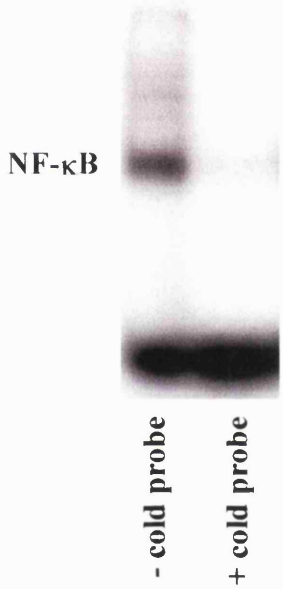


FIGURE 6. Specific binding of NF-κB probe. RAW 264.7 macrophages were activated with LPS (1μg/ml) and nuclear extract was prepared after 90 mins. NF-κB activity was assessed by EMSA with nuclear extract and ^{32}P-labelled NF-κB consensus oligonucleotide binding reactions conducted in the absence or presence of 100-fold molar excess of unlabeled (cold) NF-κB consensus oligonucleotide.

2.8 IL-6, PGE_2 and nitrite measurement

Medium samples were thawed, vortexed and centrifuged (13,793 x g, 5mins, 4°C) to remove any cells. IL-6 was measured using the Quantikine M mouse IL-6 Quantitative Colorimetric Sandwich ELISA from R and D Systems (Abingdon, UK) according to the manufacturer's instructions. Briefly, samples or standards were added to a 96-well plate which had been pre-coated with an affinity purified polyclonal antibody specific for mouse IL-6. After 2 hours incubation the wells were washed to remove any unbound substances and a HRP conjugated-polyclonal antibody specific for mouse IL-6 was added to each well and the plates incubated for a further 2 hours. The plate was then washed and an HRP substrate is added which changes colour through the action of the enzyme with the intensity of colour increasing as the concentration of IL-6 increases. The absorbance (Abs_{450}-Abs_{540}) was measured on a 96-well plate reader as before. A standard curve was prepared using mouse IL-6 standard solution provided with the kit (15.6-1000pg/ml; Figure 7). Samples were diluted in the same assay kit buffer in order that they gave a reading within the range of the standard curve.

PGE_2 was measured using the PGE_2 Colorimetric Competitive ELISA from R and D systems as instructed. Standards or samples were added to a 96-well plate coated with goat anti-mouse antibody. Alkaline phosphatase-conjugated PGE_2 and a mouse monoclonal PGE_2 antibody solution were also added to the assay mixture and the plate incubated with shaking for 1 hour. The PGE_2 in the samples or standard competes with the alkaline phosphatase-conjugated PGE_2 for binding to the mouse monoclonal antibody. During the assay this mouse monoclonal antibody binds to the coated plate. The plate was washed to remove excess conjugate or unbound sample and an alkaline phosphatase substrate solution was added which changes colour on reaction with the enzyme. If there is a high concentration of PGE_2 in the sample then it will compete strongly for binding with the antibody, reducing the levels of alkaline phosphatase-conjugated PGE_2 present. Thus the intensity of the colour will be inversely proportional to the concentration of PGE_2 in the sample. The absorbance (Abs_{405}-Abs_{570}) was measured on a 96-well plate reader as before. A standard curve was prepared using a PGE_2 standard solution provided with the kit diluted in complete

medium (39-5000pg/ml; Figure 7). Samples were diluted in complete medium in order that they gave a reading which fell on the standard curve.

Nitrite accumulation was determined by mixing equal volumes of cell culture medium and Griess Reagent (0.5% sulphanilamide, 0.05% naphthylethylenediamine dihydrochloride, 2.5% H_3PO_4). The presence of nitrite in the samples resulted in a colourimetric reaction and the absorbance (Abs_{540}-Abs_{620}) was read on a 96-well microplate reader as before. A standard curve was constructed with known concentrations of $NaNO_2$ (2.5-80μM; Figure 8), which were made up in complete medium.

STANDARD CURVES FOR IL-6 AND PGE_2 ELISAS

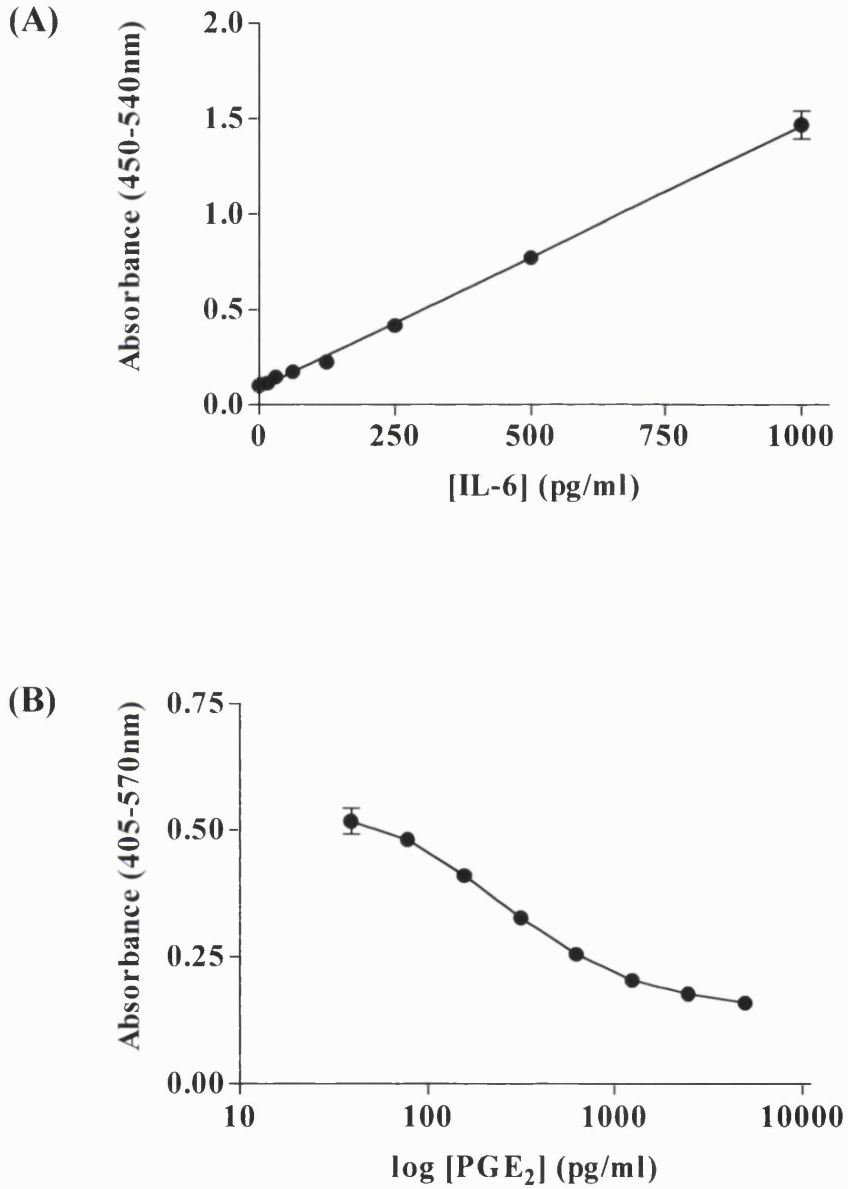


FIGURE 7. Standard curves prepared for (A) IL-6 or (B) PGE_2 ELISA kits. Increasing concentrations of IL-6 or PGE_2 were prepared and absorbance was measured using a 96-well plate reader. Data are represented as mean ± SEM absorbance ($n > 3$).

STANDARD CURVE FOR GRIESS REACTION

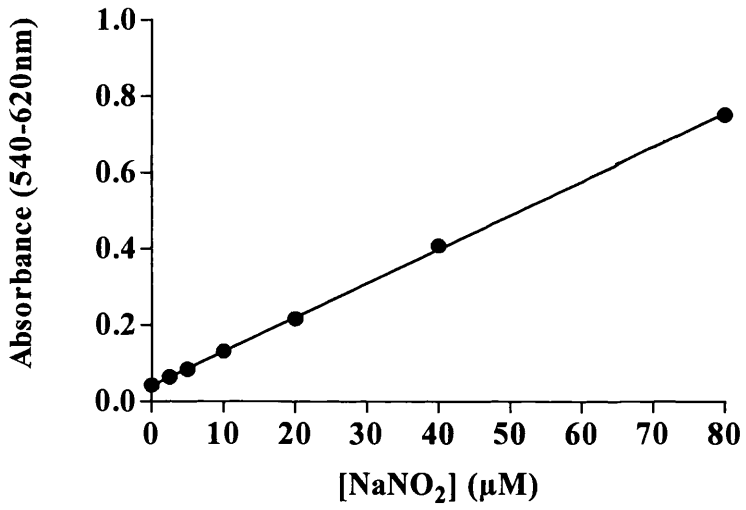


FIGURE 8. Standard curve for Griess reaction. Increasing concentrations of $NaNO_2$ were diluted in complete medium and the absorbance measured using a 96-well plate reader. Data are represented as mean ± SEM absorbance (n = 6).

2.9 eNOS TRANSFECTION EXPERIMENTS

2.9.1 eNOS cloning

The vector pComp1 containing human eNOS cDNA (4kbps) was a gift from Dr Weimin Xu (Wolfson Institute) and a pcDNA3.1 vector was obtained from Invitrogen. 1µl of each plasmid was transformed into *E. coli* K12 TOP 10 cells (Invitrogen) and plated on L-agar plates containing 50µg/ml ampicillin (Sigma). Plates were incubated at 37°C overnight after which 10 colonies were picked from each and grown at 37°C with shaking, overnight in 5ml LB medium (made with LB broth tablets, Sigma) containing 50µg/ml ampicillin. Cells were pelleted and DNA extracted using the QIAprep Spin Miniprep Kit (Qiagen, Crawley, UK) according to manufacturer's instructions. DNA was tested by restriction enzyme digest at 37°C for 1 hour with 20 units of enzyme, 1µl DNA, 1 x enzyme buffer (supplied with enzyme) in 20µl of distilled H_2O (pComp1 – EcoRI and pcDNA3.1 – Sal I; New England Biolabs, Hitchin, UK). Products were resolved by 0.7% agarose gel electrophoresis (as described previously) and sizes confirmed by comparing bands to 5µl Hyperladder 1 (200-10,000bps; Bioline, London, UK).

10µl of successful mini-prep DNA for each plasmid was digested as above with 40 units EcoRI. 10 units of calf intestinal alkaline phosphatase (New England Biolabs) were added to the pcDNA3.1 digest for the last 10mins of the digest. The digest products were separated on a 0.7% agarose gel and the eNOS insert and pcDNA3.1 vector bands were cut out. DNA was extracted from the agarose using the QIAquick Gel Extraction Kit (Qiagen) and fragments were ligated using the Lightning DNA Ligation Kit (Bioline). 1µl of each ligation reaction was transformed, plated and mini-prepped as above. The resultant DNA was digested using Xho I to determine the orientation of the eNOS insert. Clones were selected containing eNOS in both correct and incorrect orientation with the incorrect used as a negative control in transfection experiments. 1µl of mini-prep DNA was re-transformed and a single colony picked and grown in 5ml LB medium (as above) at 37°C, shaking for approximately 8 hrs. 1ml of this culture was then used to inoculate a 100ml culture, which was grown overnight as above. The DNA was extracted from cultures using the Endo-free Maxi

Plasmid Extraction Kit (Qiagen). DNA concentrations were determined by measurement of OD_{260}.

2.9.2 Transient transfection of eNOS into RAW 264.7 cells

RAW 264.7 cells were cultured as previously described. 24 hrs prior to transfection cells were plated in 6-well plates at a concentration of 400,000 cells/well in 2ml complete medium and incubated incubated overnight at 37°C in a humidified incubator containing 5% CO_2 in air. Cells were then transfected with eNOS/pcDNA3.1 (correct and incorrect orientation) using Superfect Transfection Reagent (Life Technologies) according to the manufacturer's instructions. The Superfect reagent is an activated dendrimer with branches extending from a spherical core. Each branch has a charged amino acid group at the terminal which interacts with the phosphate backbone of DNA. These molecules bind all around the plasmid until it is completely coated. This packages the complex which is transported into the cell by non-specific endocytosis. A mixture of of plasmid DNA (5μg) and Superfect reagent at a ratio of 1:2 was made in RPMI 1640 (with 25mM HEPES) and incubated for 5-10 mins at room temperature to allow complex formation. This mixture was then mixed with complete medium by pipetting. Medium was aspirated from the RAW 264.7 cells and each well was washed with PBS before addition of 1ml of the DNA/Superfect mixture. The plates were then incubated as before for 2 hours after which the transfection mixture was aspirated, cells were washed with PBS and 2ml of complete medium added per well. 24 hrs post-transfection, medium was aspirated, wells were washed with PBS and replenished with 2ml complete medium. The cells were then activated with LPS (1μg/ml) and after 24 hrs protein extracts were prepared as described previously (whole cell extract).

2.10 MEASUREMENT OF NO RELEASE USING NO ELECTRODE

The release of NO by DEA-NO was measured using an NO electrode (Iso-NO, World Precision Instruments, Stevenage, UK). The electrode chamber is a gas-tight vessel which is maintained at 37°C. NO diffuses through a polymeric membrane into the

electrode chamber and is oxidised at the electrode. This results in a redox current which is proportional to the concentration of NO in the sample. Initially the electrode was calibrated with known concentrations of $NaNO_2$ under reducing conditions (KI/H_2SO_4) and a standard curve was plotted (Figure 9). Following calibration 1ml of complete medium was added containing 1 x 10^6 RAW 264.7 cells and this was maintained in the chamber with constant stirring. Following the addition of known concentrations of DEA-NO by syringe, peak NO release was measured. The measurements from standards and samples were analysed using the Duo 18 recording system and software (World Precision Instruments) and release of NO from DEA-NO determined from the standard curve.

CALIBRATION CURVE FOR NO ELECTRODE

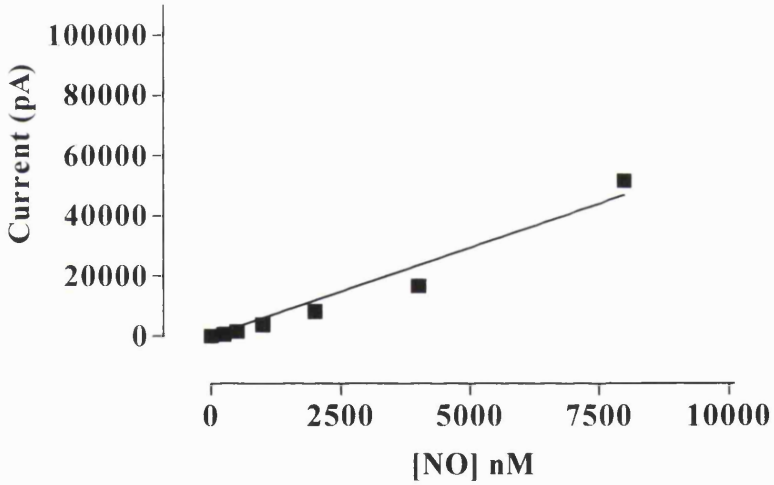


FIGURE 9. Calibration of the NO electrode. Increasing concentrations of $NaNO_2$ (0-8000nM) were added to the NO electrode and the current generated was measured. Data are represented as mean ± SEM current ($n \geq 4$).

2.11 DATA ANALYSIS

All statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, USA). Densitometric analyses were performed using NIH Image. All data are plotted graphically as mean values with vertical bars representing standard error of the mean (SEM). A Student's t-test was used to assess differences between experimental conditions. A probability (p) value of ≤ 0.05 was taken as an appropriate level of significance.

CHAPTER THREE

RESULTS

3. RESULTS

3.1 CHARACTERISATION OF RAW 264.7 CELLS

3.1.1 Optimisation of LPS stimulation

RAW 264.7 cells were activated with *E. coli* or *S. typhosa* LPS (1μg/ml) and nitrite concentrations in the cell medium were measured after 24 hrs (Table 1). *S. typhosa* LPS was selected for further experiments as it gave a greater concentration of nitrite.

The experimental media were tested in a similar manner (Table 1) and cells were found to produce more nitrite in RPMI 1640 than DMEM. Additionally, the use of RPMI 1640 supplemented with 25mM HEPES resulted in a higher concentration of nitrite than with RPMI 1640 alone and was selected for further experimentation.

3.1.2 Concentration response to LPS

Activation of RAW 264.7 cells with increasing concentrations of LPS (0-10μg/ml) led to a concentration-dependent increase in iNOS protein expression and activity as observed after 24 hrs (Figure 10). The peak protein expression and nitrite production were apparent at a concentration of 1μg/ml LPS and this concentration was selected for future studies.

OPTIMISATION OF LPS-STIMULATION OF RAW 264.7 CELLS

CONDITION	NITRITE (μM)
E. coli LPS in RPMI 1640	6.80 ± 0.788
S. typhosa LPS in RPMI 1640	12.22 ± 0.483
S. typhosa LPS in DMEM	6.71 ± 0.069
S. typhosa LPS in RPMI 1640 (+25mM HEPES)	16.03 ± 0.370

TABLE 1. Accumulation of nitrite in the culture medium of RAW 264.7 macrophages activated with *E. coli* or *S. typhosa* LPS (1μg/ml) in different media. Nitrite concentrations were measured by Griess reaction 24 hrs after activation. Data are represented as the mean ± SEM nitrite production under each condition (n = 3).

CONCENTRATION RESPONSE TO LPS IN RAW 264.7 CELLS

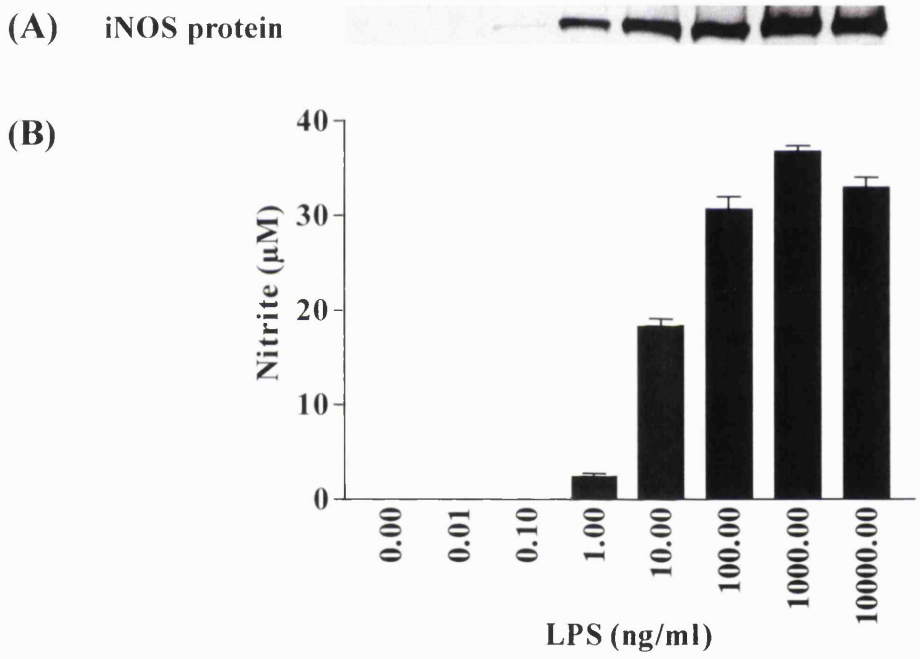


FIGURE 10. Expression of iNOS protein and nitrite production in RAW 264.7 macrophages activated with increasing concentrations of LPS (0-10,000ng/ml). Protein expression was analysed by Western blot (A), and nitrite concentrations measured by Griess reaction (B) after 24 hrs. Data are represented as the mean ± SEM nitrite production under each condition (n = 3).

3.1.3. Time course of iNOS and COX-2 expression

RAW 264.7 cells were activated with 1μg/ml LPS and samples taken at different time points (0-48 hrs) to determine iNOS (11) and COX-2 (Figure 12) expression and activity. A transient expression pattern was observed for each protein after activation with LPS. There was no expression of iNOS or COX-2, as determined by Western blot, in unstimulated cells (data not shown).

iNOS mRNA was first expressed after 3 hrs with a peak at 24 hrs and a down regulation such that it was almost absent after 48 hrs. A similar pattern of expression was observed with iNOS protein, which first appeared at 6 hrs and increased until reaching a maximum between 12 and 24 hrs before decreasing at 48 hrs. The production of nitrite was detectable 9 hrs after activation and continued to rise over the entire 48 hrs of the experiment.

The expression of COX-2 mRNA occurred slightly earlier than that of iNOS with a PCR product detectable after 1 hr. The mRNA expression then peaked between 12 and 24 hrs and was decreasing by 48 hrs. Despite the earlier appearance of COX-2 mRNA, the expression of COX-2 protein followed the same time course as iNOS with an increase between 6 and 24 hrs and a marked reduction at 48 hrs. PGE_2 was apparent from 3 hrs following activation and continued to increase throughout the 48 hrs.

The viability of the cells was measured by trypan blue exclusion at each of the time points. As seen in Figure 13, the cells remained viable throughout the experiment and LPS activation did not result in significant cell death.

TIME COURSE OF iNOS EXPRESSION AND ACTIVITY IN RAW 264.7 CELLS

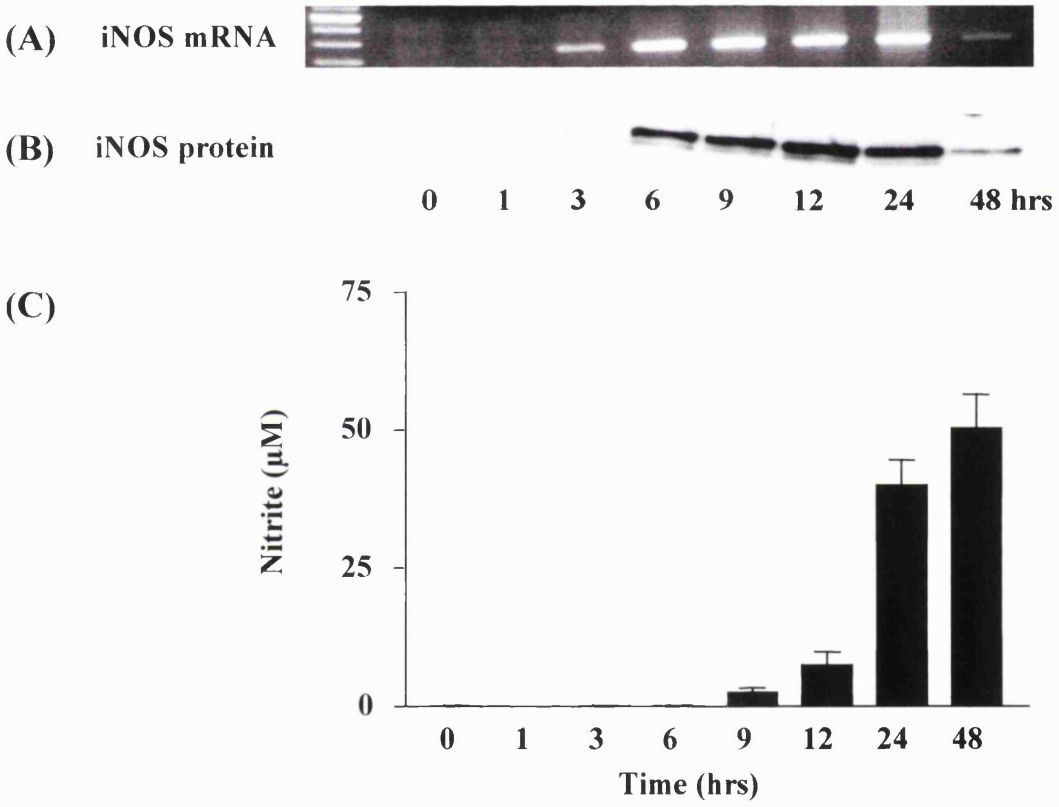


FIGURE 11. Expression of iNOS mRNA and protein and nitrite production in RAW 264.7 macrophages activated with LPS (1μg/ml). Expression was analysed by RT-PCR (A) and Western blot (B) and nitrite was measured by Griess reaction (C). Data are represented as the mean ± SEM nitrite production under each condition (n = 3).

TIME COURSE OF COX-2 EXPRESSION AND ACTIVITY IN RAW 264.7 CELLS

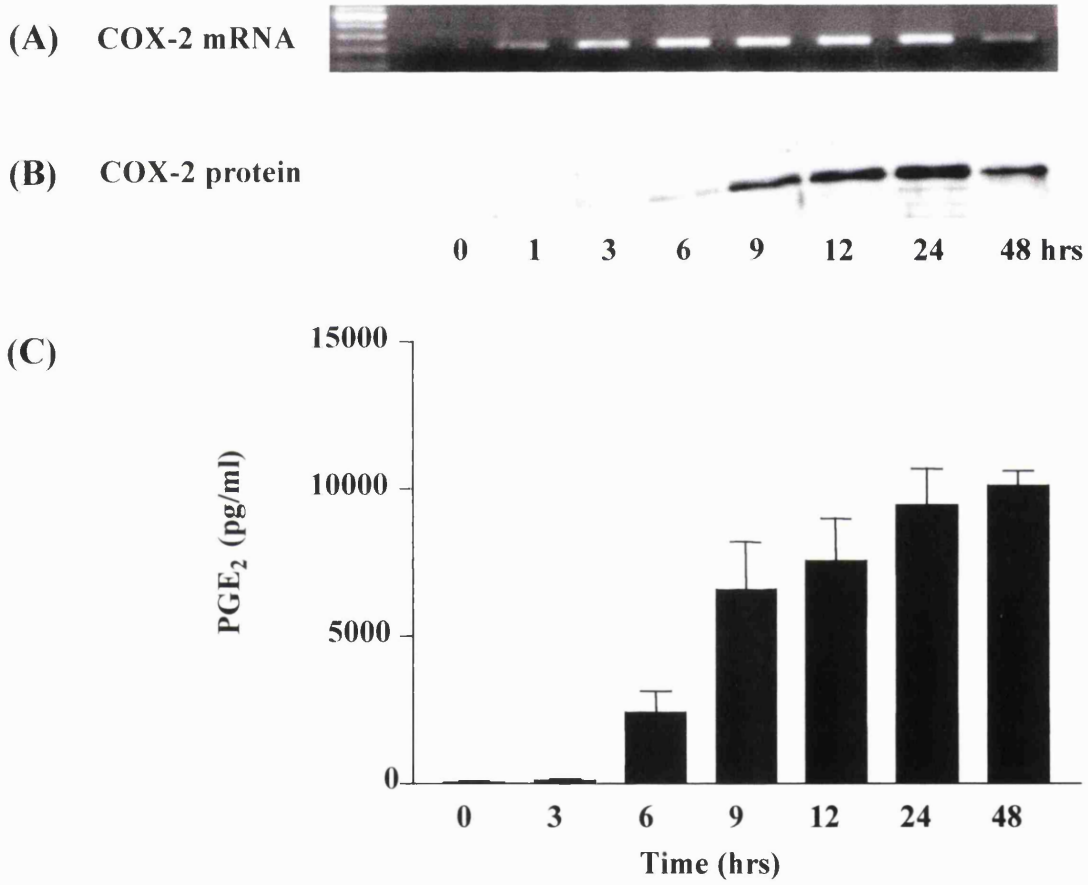


FIGURE 12. Expression of COX-2 mRNA and protein and PGE_2 production in RAW 264.7 macrophages activated with LPS (1μg/ml). Expression was analysed by RT-PCR (A) and Western blot (B) and PGE_2 was measured by ELISA (C). Data are represented as the mean ± SEM PGE_2 production under each condition (n = 3).

VIABILITY OF RAW 264.7 CELLS FOLLOWING LPS ACTIVATION

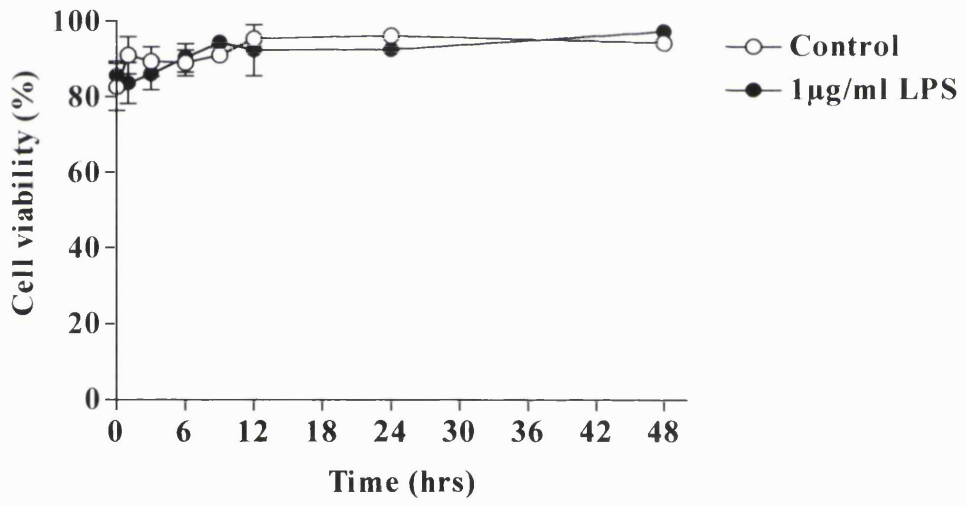


FIGURE 13. Viability of RAW 264.7 cells in the presence or absence of LPS (1µg/ml) over 48 hrs. Viability was measured by trypan blue exclusion. Data are represented as the mean ± SEM viability expressed as a percentage of live cells (n > 3).

3.2 EFFECT OF THE iNOS INHIBITOR, 1400W, ON LPS-INDUCED iNOS AND COX-2 EXPRESSION IN RAW 264.7 CELLS

In order to study the effect of blocking endogenous NO production on iNOS and COX-2 expression, RAW 264.7 cells were activated with 1μg/ml LPS in the presence and absence of the iNOS-specific inhibitor, 1400W (10μM).

Nitrite production was almost completely abolished in the presence of 1400W, indicating that iNOS activity was suppressed by the inhibitor (Figure 14). At earlier time points (0-6 hrs), 1400W slightly decreased the expression of iNOS protein and mRNA (Figure 15). However, the peak expression of iNOS protein, as measured at 24 hrs, was marginally increased by 1400W and the protein expression after 48 hrs was prolonged, with approximately 100% more expression remaining in the presence of 1400W. The effect on iNOS mRNA was similar, with a greater, more prolonged expression of mRNA in the presence of 1400W.

In contrast, levels of COX-2 protein and mRNA were markedly reduced at each time point in cells treated with 1400W as compared with control cells, apart from 48 hrs when the control mRNA levels were equivalent to those in the presence of 1400W (Figure 16). The peak expression of COX-2 protein at 24 hrs was reduced by 50% by 1400W but at 48 hrs the protein levels under each condition were not significantly different.

EFFECT OF THE iNOS INHIBITOR, 1400W ON LPS-INDUCED NITRITE PRODUCTION IN RAW 264.7 CELLS

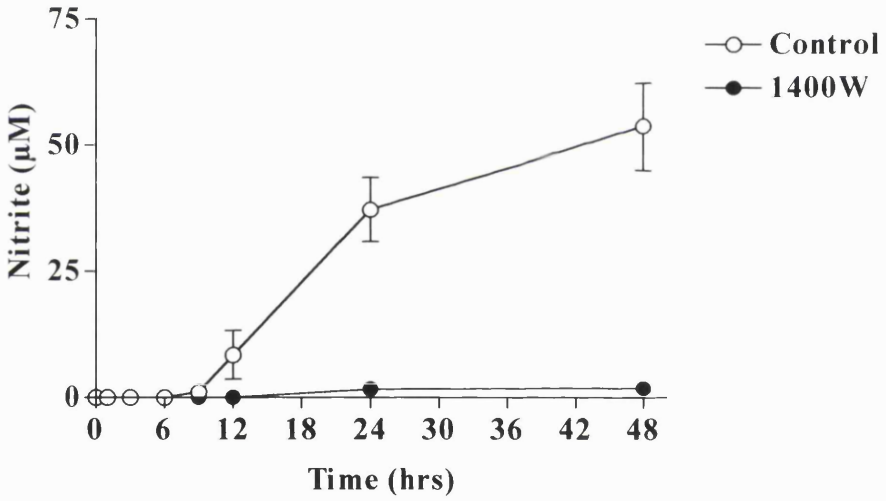


FIGURE 14. Accumulation of nitrite in the culture medium of RAW 264.7 cells activated with LPS (1μg/ml) under control conditions or in the presence of 1400W (10μM). Data are represented as the mean ± SEM nitrite production under each condition (n = 3).

EFFECT OF THE iNOS INHIBITOR, 1400W, ON LPS-INDUCED iNOS EXPRESSION IN RAW 264.7 CELLS

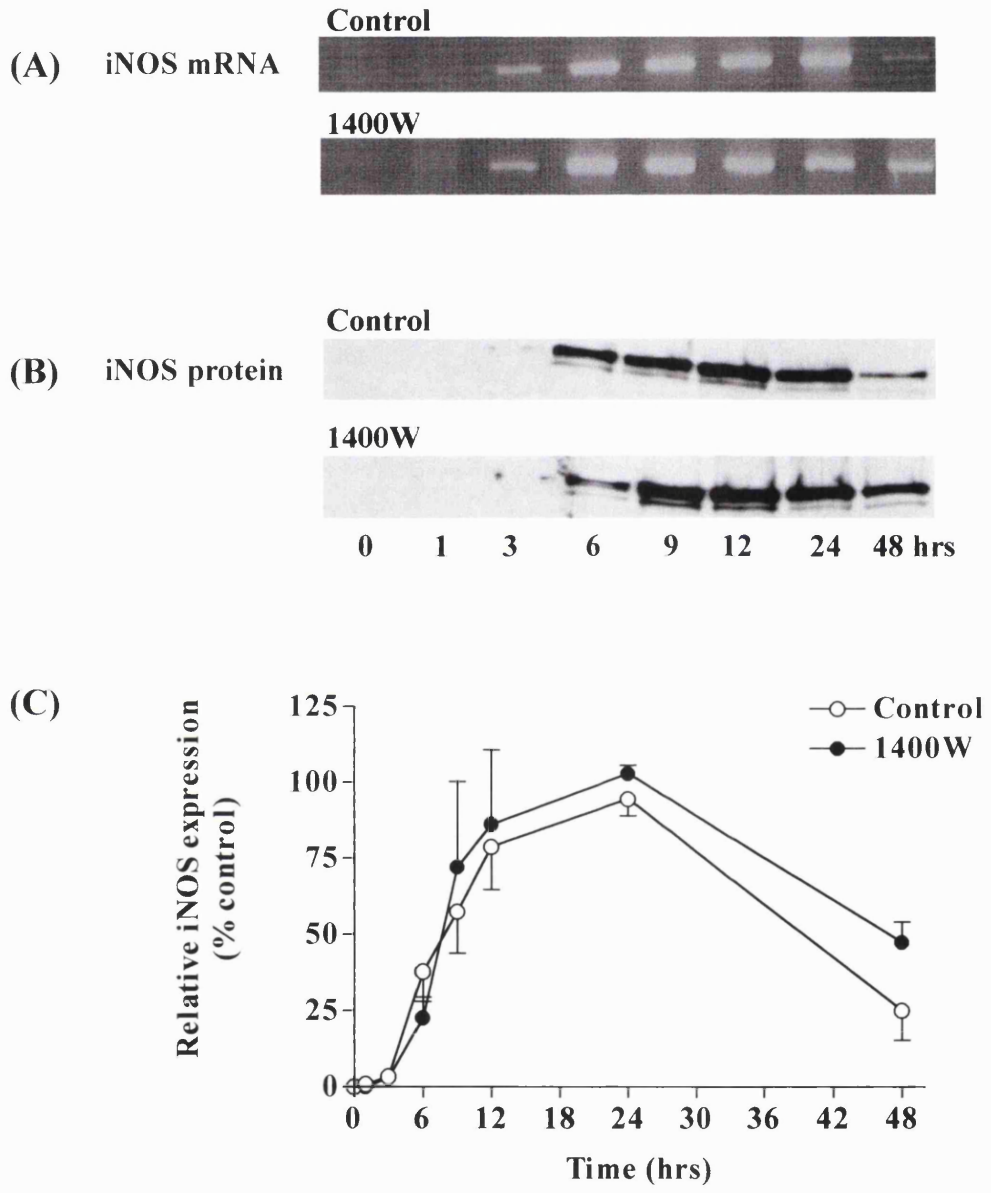


FIGURE 15. Expression of iNOS mRNA and protein in RAW 264.7 macrophages activated with LPS (1μg/ml) under control conditions or in the presence of 1400W (10μM). mRNA expression was analysed by RT PCR (A). Protein expression was analysed by Western blot (B) and bands were quantified by densitometry (C). Data are represented as the mean ± SEM density, expressed as a percentage of peak protein under control conditions (n = 3).

EFFECT OF THE iNOS INHIBITOR, 1400W, ON LPS-INDUCED COX-2 EXPRESSION IN RAW 264.7 CELLS

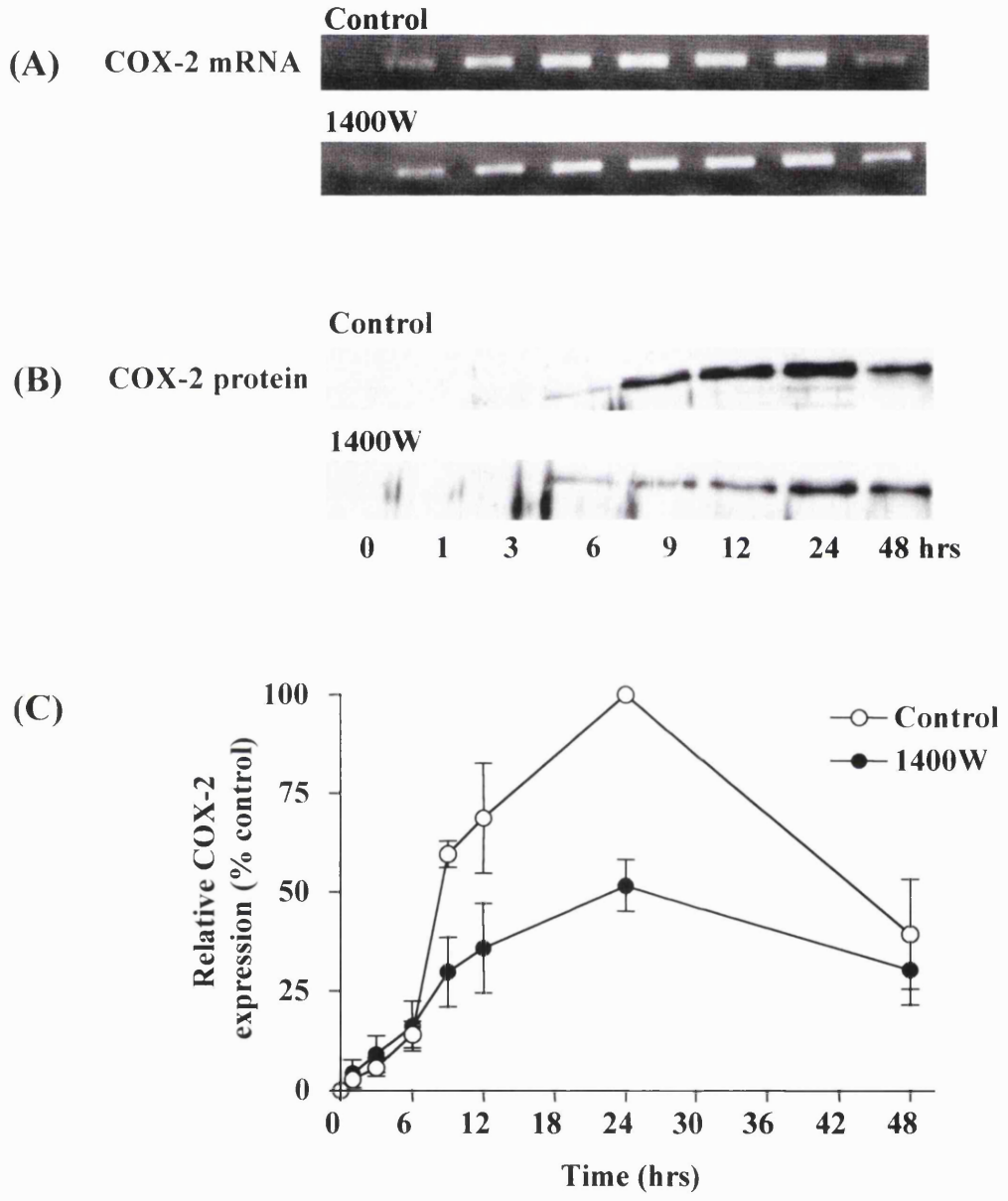


FIGURE 16. Expression of COX-2 mRNA and protein in RAW 264.7 macrophages activated with LPS (1μg/ml) under control conditions or in the presence of 1400W (10μM). mRNA expression was analysed by RT PCR (A). Protein expression was analysed by Western blot (B) and bands were quantified by densitometry (C). Data are represented as the mean ± SEM density, expressed as a percentage of peak protein under control conditions (n = 3).

3.3 EFFECT OF L-ARGININE-FREE MEDIUM ON PRO-INFLAMMATORY PROTEIN EXPRESSION IN RAW 264.7 CELLS

3.3.1 iNOS and COX-2 expression

L-arginine free medium was also used to prevent endogenous NO production as iNOS activity is inhibited in the absence of an external supply of substrate (Assreuy & Moncada, 1992). In initial experiments, the RAW 264.7 cells were seeded in L-arginine free medium for 24 hrs prior to activation with 1μg/ml LPS, after which iNOS and COX-2 mRNA and protein were measured. This protocol led to a complete inhibition of iNOS and COX-2 protein expression with almost no protein detected in the LPS-activated cells (Figure 17). Therefore, the experimental protocol was changed such that the cells were only transferred into L-arginine free medium at the time of activation with LPS. Under these conditions, inflammatory protein expression was still observed, but nitrite production was abolished, indicating that the lack of L-arginine was selectively preventing iNOS activity (Figure 18).

In L-arginine free medium there was a marked shift in the profile of iNOS expression (Figure 19). iNOS mRNA levels were reduced at earlier time points (0-24 hrs) while at later time points (72 and 96 hrs) higher levels of mRNA remained. Appearance of iNOS protein was delayed such that levels were significantly reduced (0-24 hrs) with peak expression occurring after 48 hrs (as opposed to 12 hrs in the control); maximum protein expression in L-arginine free medium was significantly reduced as compared to control (75.4 ± 7.8%; $n \geq 3$; $p < 0.05$). At later time points substantial levels of protein remained as compared with the more rapid down regulation in the control cells.

A similar phenomenon was observed with COX-2 mRNA and protein expression (Figure 20). At earlier time points (0-24 hrs) mRNA levels were reduced while at later time points (48-72 hrs) higher levels of mRNA remained in the absence of L-arginine. COX-2 protein expression was delayed initially with no expression occurring till 9 hrs and peak expression at 48 hrs. The peak expression was decreased to 80.4 ± 12.6% ($n \geq 3$; $p < 0.05$) as compared with control.

In summary, the blocking of endogenous NO production by removal of L-arginine delayed the initial onset of iNOS and COX-2 expression yet once protein had been produced, the expression was prolonged.

3.3.2 PGE_2 production

The concentration of PGE_2 was measured at each time point and, while initially the slower increase in PGE_2 accumulation mirrored the changes observed in protein expression, at later time points PGE_2 concentration failed to rise to the levels obtained in control cells (Figure 21).

3.3.3 IL-6 production

The accumulation of a third inflammatory protein, IL-6, was measured when endogenous NO production was blocked using L-arginine free medium (Figure 22). Under control conditions, the LPS-stimulated increase in concentration of IL-6 was first apparent after 6 hrs with production continuing over the whole time course of the experiment. In L-arginine free medium, the production of IL-6 was slowed during the initial 12 hrs of the experiment while after this time point the concentration of IL-6 exceeded that of the control cells.

EFFECT OF INHIBITION OF ENDOGENOUS NO PRODUCTION ON iNOS AND COX-2 EXPRESSION IN RAW 264.7 CELLS

(L-ARGININE REMOVAL FOR 24 HRS PRIOR TO ACTIVATION)

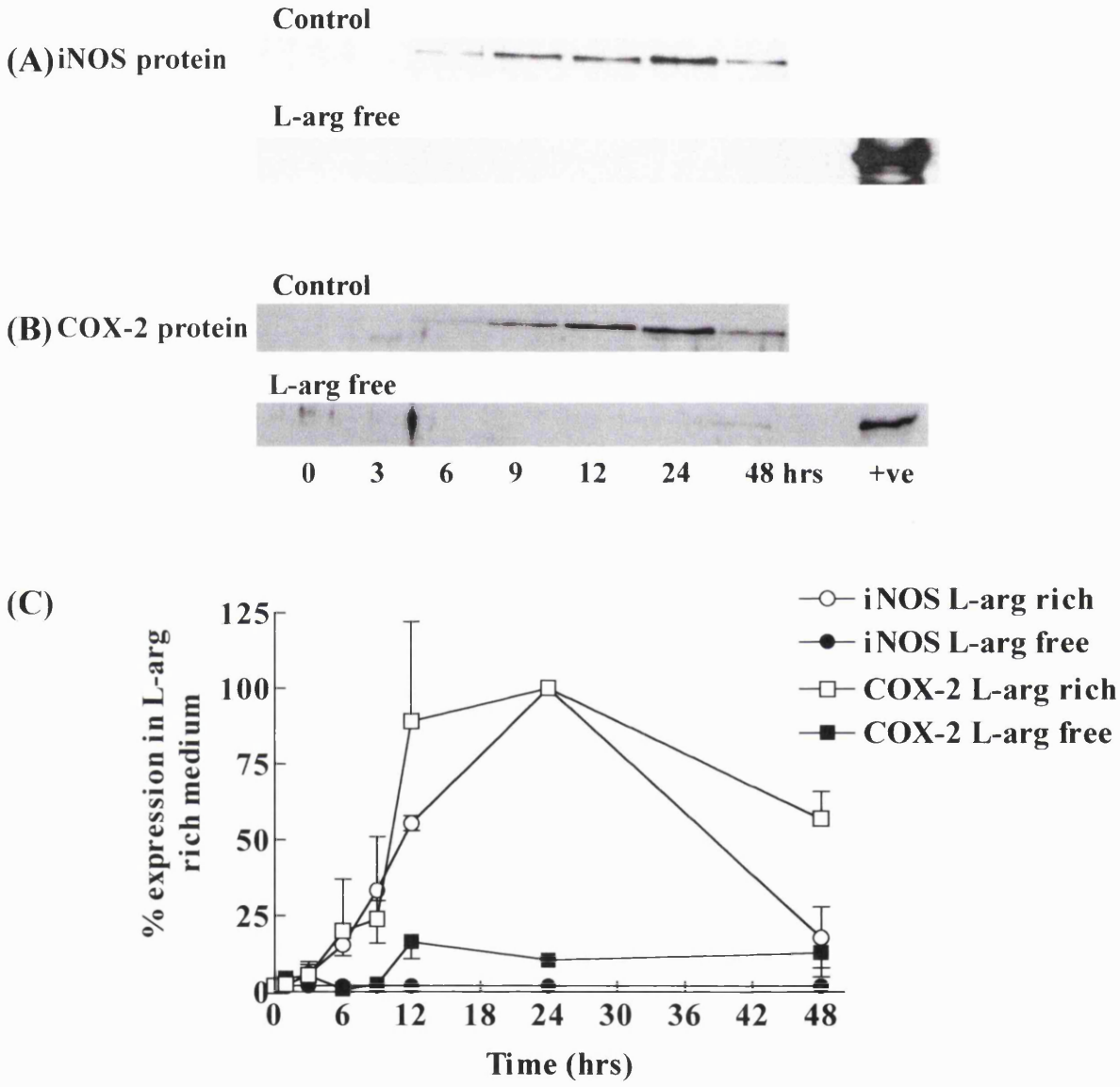


FIGURE 17. Expression of iNOS and COX-2 protein in RAW 264.7 macrophages activated with LPS (1μg/ml) under control conditions or in the presence of L-arginine (L-arg)-free medium (pre-incubation for 24 hrs in L-arg-free medium). Protein expression was analysed by Western blot (A and B), and bands were quantified by densitometry (C). Positive (+ve) control was commercially supplied. Data are represented as the mean ± SEM density, expressed as a percentage of the peak protein under control conditions (n = 3).

EFFECT OF L-ARGININE FREE MEDIUM AND L-NAME ON NITRITE PRODUCTION IN RAW 264.7 CELLS

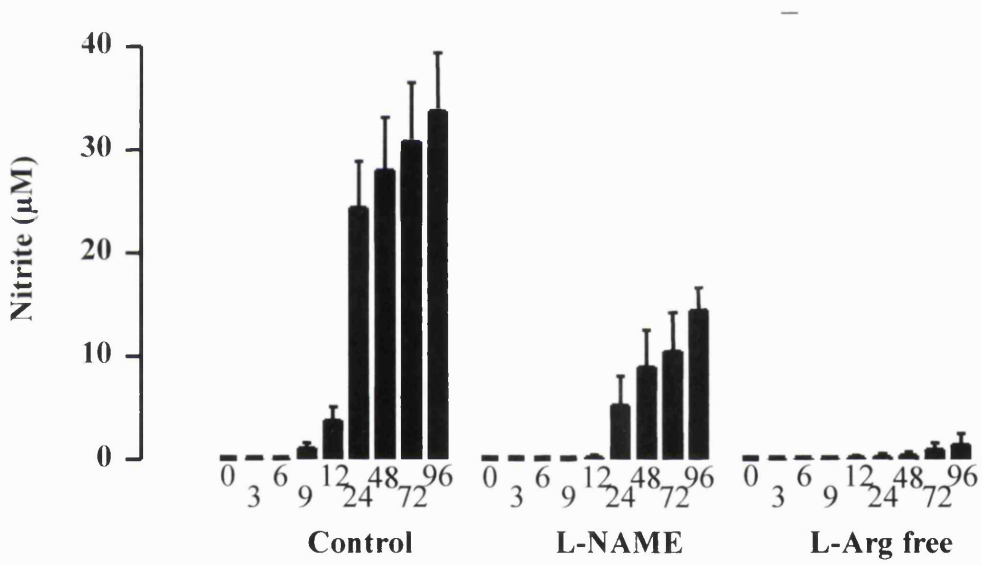
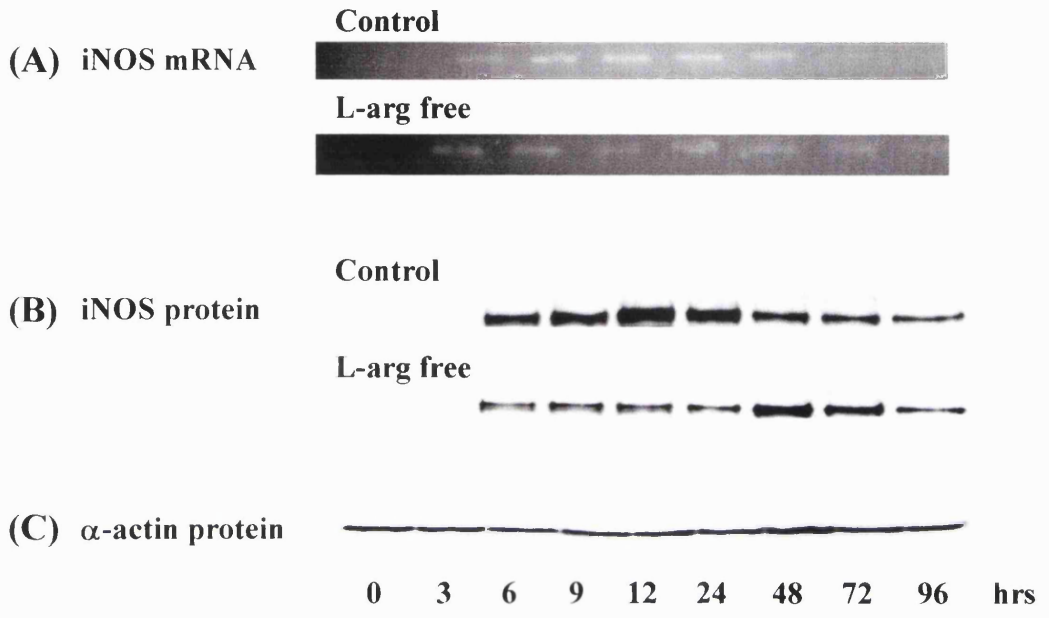


FIGURE 18. Accumulation of nitrite in the culture medium of RAW 264.7 cells activated with LPS (1μg/ml) under control conditions or in the presence of L-NAME (1mM) or L-arginine (L-arg)-free medium. Nitrite was measured by Griess reaction over 96 hrs. Data are represented as the mean ± SEM nitrite production under each condition ($n > 3$).

EFFECT OF INHIBITION OF ENDOGENOUS NO PRODUCTION ON iNOS EXPRESSION IN RAW 264.7 CELLS



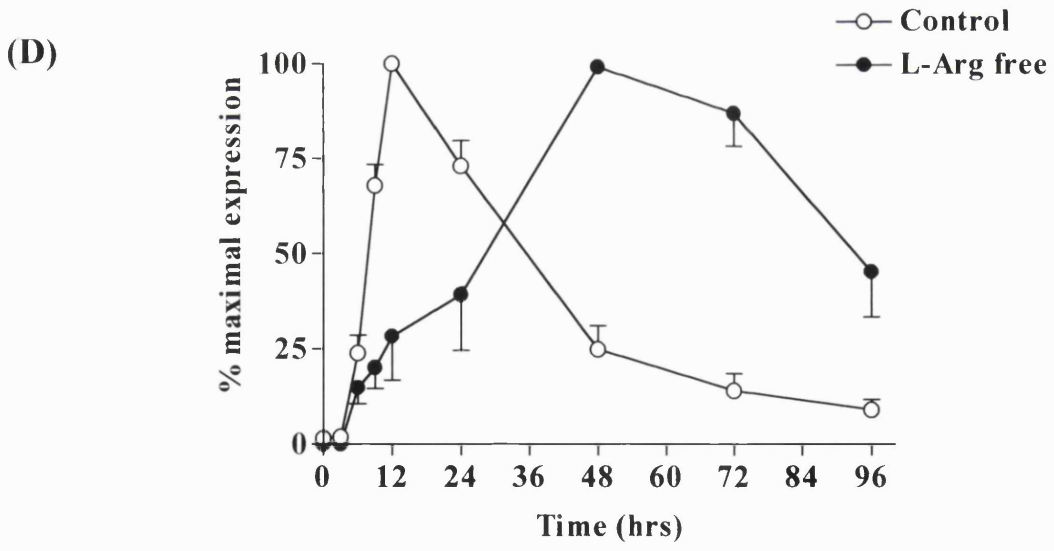


FIGURE 19. Expression of iNOS mRNA and protein in RAW 264.7 macrophages activated with LPS (1μg/ml) under control conditions or in the presence of L-arginine (L-arg)-free medium. mRNA expression was analysed by RT-PCR (A). iNOS (B) and α-actin (C) protein expression were analysed by Western blot. iNOS protein bands were quantified by densitometry (D) and data are represented as the mean ± SEM density, expressed as a percentage of the peak protein under each condition ($n > 3$).

EFFECT OF INHIBITION OF ENDOGENOUS NO PRODUCTION ON COX-2 EXPRESSION IN RAW 264.7 CELLS

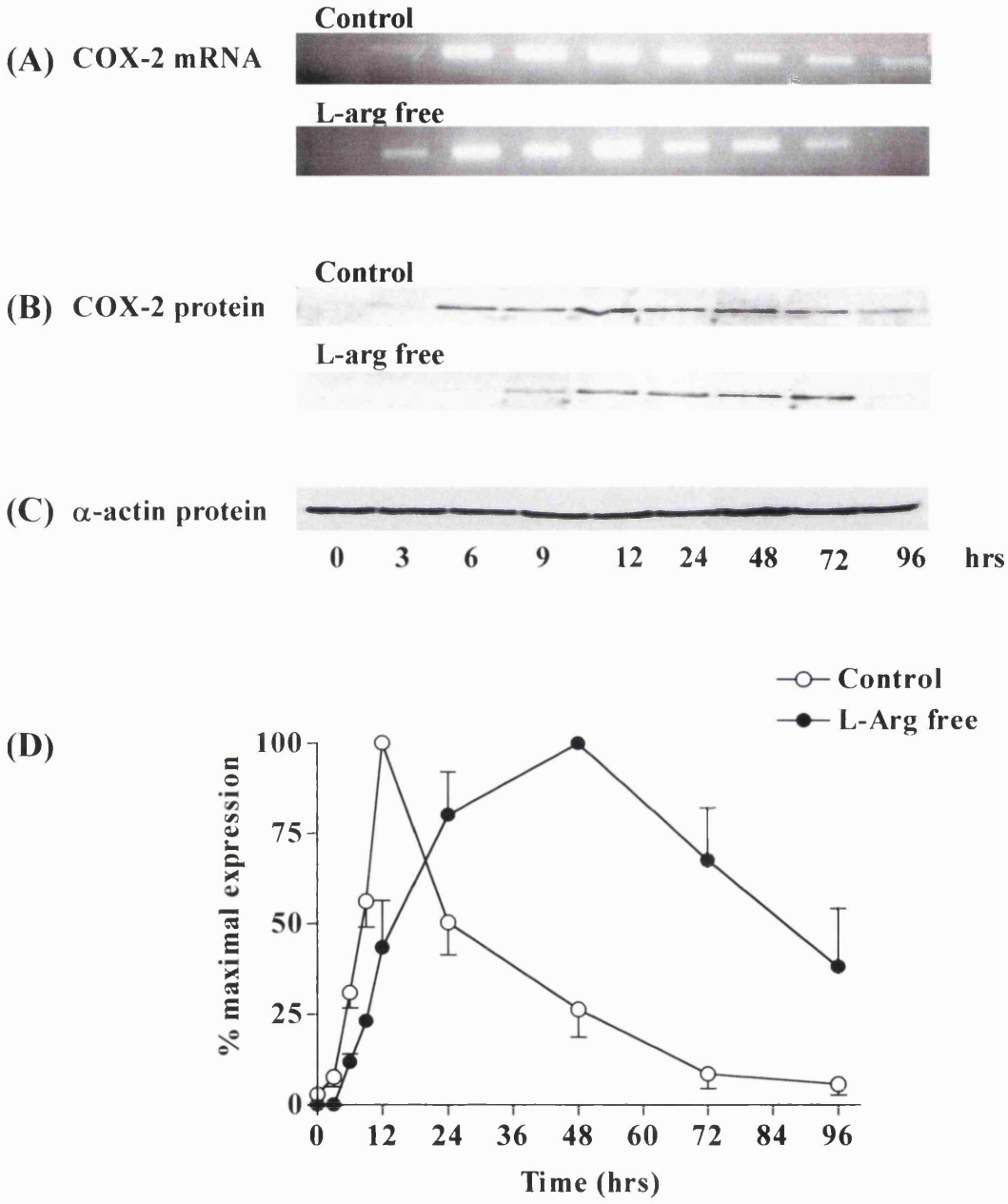


FIGURE 20. Expression of COX-2 mRNA and protein in RAW 264.7 macrophages activated with LPS (1μg/ml) under control conditions or in the presence of L-arginine (L-arg)-free medium. mRNA expression was analysed by RT-PCR (A). COX-2 (B) and α-actin (C) protein expression were analysed by Western blot. COX-2 protein bands were quantified by densitometry (D) and data are represented as the mean ± SEM density, expressed as a percentage of the peak protein under each condition (n > 3).

EFFECT OF INHIBITION OF ENDOGENOUS NO PRODUCTION ON PGE_2 IN RAW 264.7 CELLS

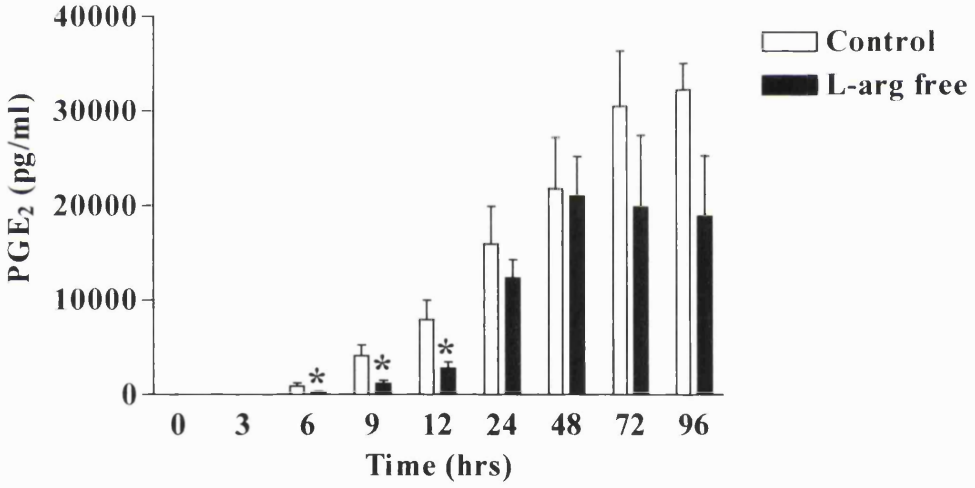


FIGURE 21. Accumulation of PGE_2 in the culture medium of RAW 264.7 macrophages activated with LPS (1μg/ml) under control conditions or in the presence of L-arginine (L-arg)-free medium. PGE_2 was measured by ELISA. Data are represented as the mean ± SEM PGE_2 production under each condition (n > 3); * $p <$ 0.05 vs control.

EFFECT OF INHIBITION OF ENDOGENOUS NO PRODUCTION ON IL-6 IN RAW 264.7 CELLS

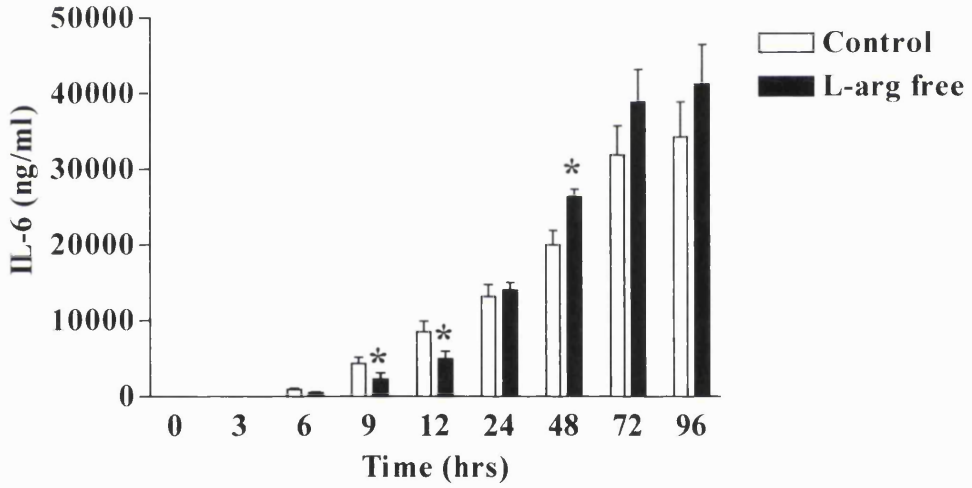


FIGURE 22. Accumulation of IL-6 in the culture medium of RAW 264.7 macrophages activated with LPS (1μg/ml) under control conditions or in the presence of L-arginine (L-arg)-free medium. IL-6 was measured by ELISA. Data are represented as the mean ± SEM IL-6 concentration under each condition (n > 3); * $p <$ 0.05 vs control.

3.4 EFFECT OF THE NON-SELECTIVE NOS INHIBITOR, L-NAME, ON PRO-INFLAMMATORY PROTEIN EXPRESSION IN RAW 264.7 CELLS

3.4.1 iNOS and COX-2 expression

To confirm that the effects observed with L-arginine free medium were due specifically to the inhibition of NO synthesis, cells were activated with LPS (1μg/ml) in the presence of the non-selective NOS inhibitor, L-NAME. As with the L-arginine free medium, the presence of L-NAME (1mM) led to a rightwards shift in the profile of iNOS (Figure 23) and COX-2 (Figure 24) expression.

From 0 to 12 hrs iNOS mRNA levels were lower in the presence of L-NAME and peak expression occurred at 24 hrs (as opposed to 9 hrs in control). iNOS protein expression was slowed in the presence of L-NAME with peak expression occurring at 24 hrs (as compared with 12 hrs under control conditions). At later time points, iNOS expression was prolonged (48 to 96 hrs) with approximately 100% more protein remaining at 96 hrs in the presence of L-NAME.

Levels of COX-2 mRNA were higher at earlier time points (0 to 12 hrs) in the presence of L-NAME and at later time points mRNA expression was prolonged. COX-2 protein expression was slowed initially by L-NAME in that peak expression occurred at 24 hrs instead of 12 hrs. At later time points (48 and 72 hrs) protein expression was maintained above control levels.

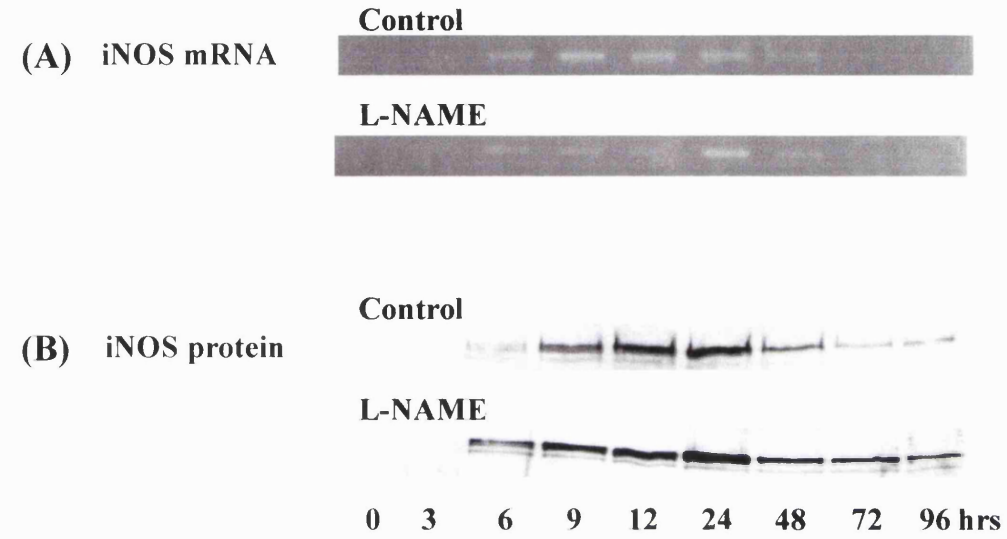
In sum, iNOS and COX-2 protein expression was initially delayed but later prolonged in the presence of L-NAME. However, this shift was not so pronounced as in L-arginine free medium.

3.4.2 IL-6 production

In the presence of L-NAME (1mM) the level of IL-6 observed up to 24 hrs was below the control values while after this point IL-6 levels in L-NAME-treated cells exceeded

control values (Figure 25). This was a similar shift to that observed with L-arginine free medium.

EFFECT OF THE NOS INHIBITOR, L-NAME, ON iNOS EXPRESSION IN RAW 264.7 CELLS



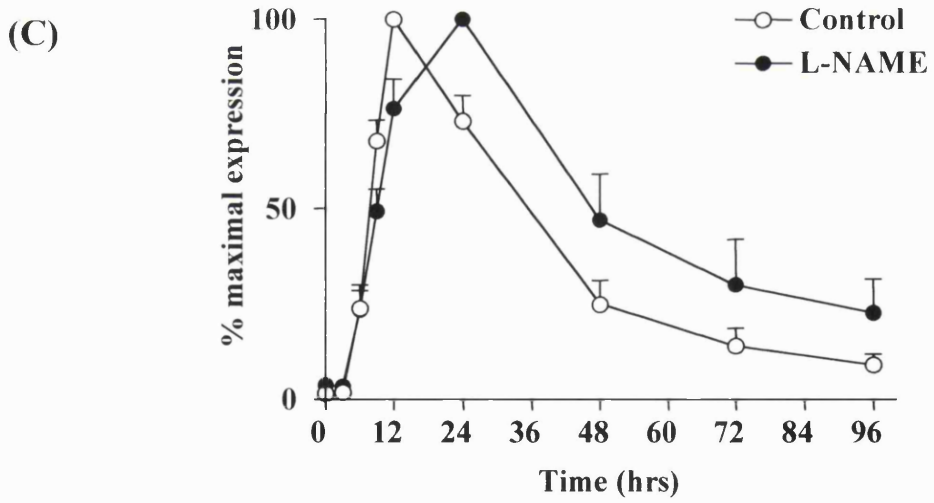


FIGURE 23. Expression of iNOS mRNA and protein in RAW 264.7 macrophages activated with LPS (1μg/ml) under control conditions or in the presence of L-NAME (1mM). mRNA expression was analysed by RT-PCR (A). Protein expression was analysed by Western blot (B), and bands were quantified by densitometry (C). Data are represented as the mean ± SEM density, expressed as a percentage of the peak protein under each condition (n > 3).

EFFECT OF THE NOS INHIBITOR, L-NAME, ON COX-2 EXPRESSION IN RAW 264.7 CELLS

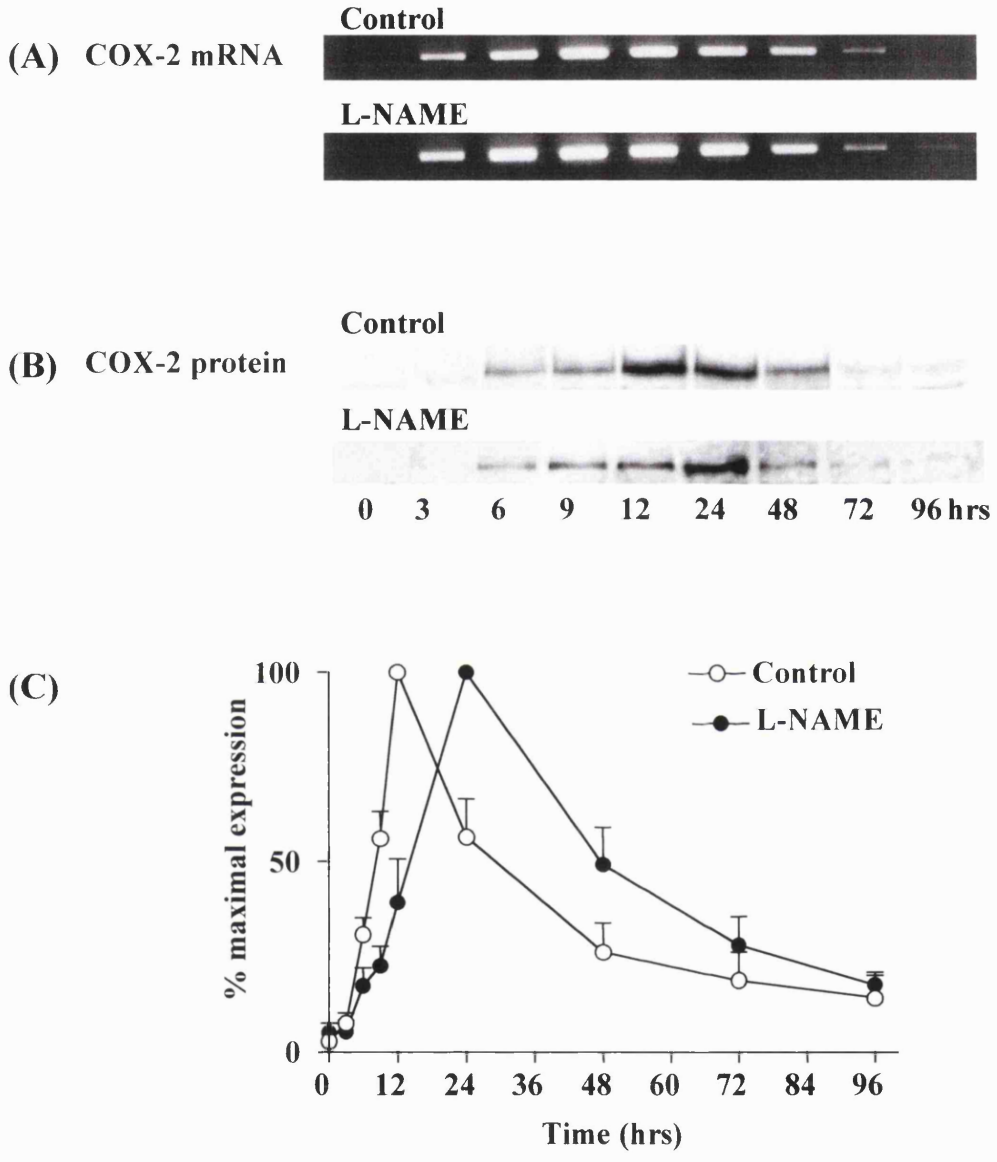


FIGURE 24. Expression of COX-2 mRNA and protein in RAW 264.7 macrophages activated with LPS (1μg/ml) under control conditions or in the presence of L-NAME (1mM). mRNA expression was analysed by RT-PCR (A). Protein expression was analysed by Western blot (B), and bands were quantified by densitometry (C). Data are represented as the mean ± SEM density, expressed as a percentage of the peak protein under each condition ($n > 3$).

EFFECT OF THE NOS INHIBITOR, L-NAME ON IL-6 IN RAW 264.7 CELLS

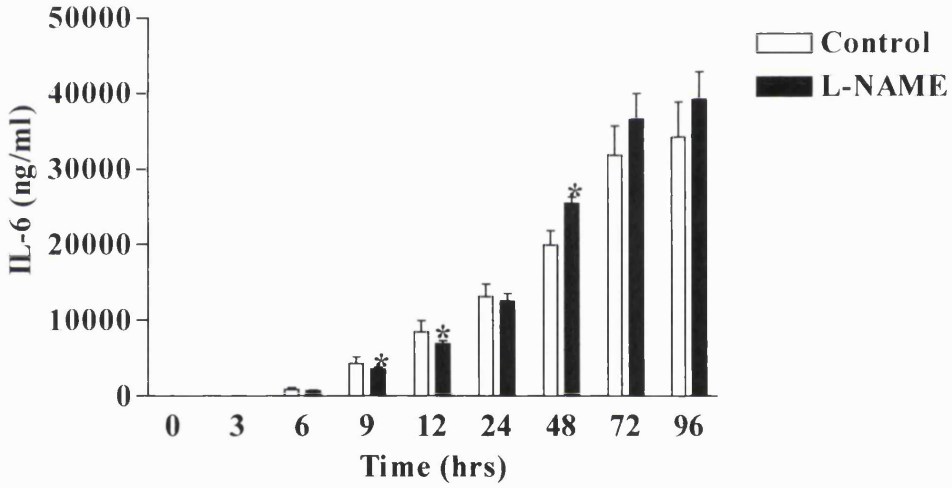


FIGURE 25. Accumulation of IL-6 in the culture medium of RAW 264.7 macrophages activated with LPS (1μg/ml) under control conditions or in the presence of L-NAME (1mM). IL-6 was measured by ELISA over 96 hrs. Data are represented as the mean ± SEM IL-6 concentration under each condition (n > 3); * $p < 0.05$ vs control.

3.4.3 Nitrite production

Since the effect of L-NAME on pro-inflammatory protein expression was significantly less pronounced than L-arginine free medium, nitrite accumulation was measured under each condition to assess the extent of inhibition of endogenous NO synthesis (Figure 18). Under control conditions nitrite concentration increased steadily over the time course of the experiment to a peak production of ~35μM. When the cells were activated in L-arginine free medium nitrite production was almost completely abolished. In comparison, after treatment with L-NAME (1mM) concentrations of nitrite reached a peak of ~15μM after 96 hrs, although this was also significantly reduced compared to the control concentration. The presence of L-NAME alone did not result in the accumulation of any nitrite over 96 hrs (data not shown). Therefore, L-NAME did not efficiently block endogenous NO production, as compare with L-arginine free medium, over the entire time course of the experiment.

3.5 EFFECT OF INHIBITION OF ENDOGENOUS NO PRODUCTION AT DIFFERENT TIME POINTS AFTER ACTIVATION IN RAW 264.7 CELLS

3.5.1 iNOS expression

In order to study the effects of blocking endogenous NO production on the early and late stages of macrophage activation, L-NAME (1mM) was added at different time points after LPS-stimulation and the effect on iNOS protein expression was assessed (Figure 27).

Initially, L-NAME was added concomitantly with LPS and iNOS protein expression was examined after 9 hrs. Under this condition iNOS expression was reduced as compared to control levels. In a parallel experiment, cells were activated with LPS but L-NAME was added 9 hrs after activation. In this case, the amount of iNOS protein observed 24 hrs post-activation was increased in the presence of the NOS inhibitor.

3.6 EFFECT OF NO ON NF-κB IN RAW 264.7 CELLS

3.6.1 Effect of endogenous NO on NF-κB activity

To determine whether the effects of inhibition of endogenous NO production on iNOS, COX-2 and IL-6 expression were the result of modification of NF-κB activity, EMSAs were carried out on nuclear extracts made from cells activated in the presence of L-NAME (1mM). Under control conditions the activation of NF-κB by LPS (1μg/ml) reached a peak 0.5-2 hrs after stimulation and returned to control levels after 24 hrs (Figure 26). When cells were activated with LPS (1μg/ml) in the presence of L-NAME (1mM) there was a decrease in NF-κB DNA-binding activity after 90 mins. In contrast, addition of L-NAME 9 hrs after LPS led to a slight increase in the NF-κB activity remaining after 24 hrs (Figure 27).

TIME COURSE OF NF-κB ACTIVITY IN RAW 264.7 CELLS

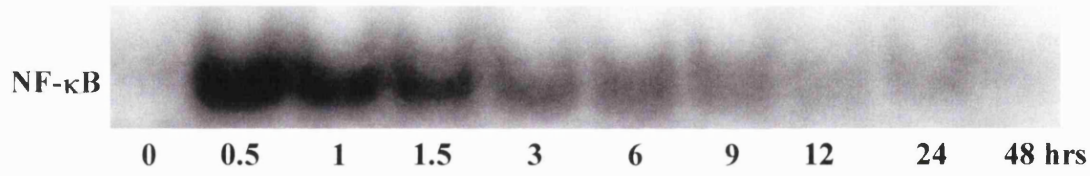


FIGURE 26. Activity of the transcription factor NF-κB in RAW 264.7 macrophages activated with LPS (1μg/ml). NF-κB activity was measured by EMSA over 48 hrs. Data are representative of at least three separate experiments.

EFFECT OF INHIBITION OF ENDOGENOUS NO PRODUCTION ON iNOS EXPRESSION AND NF-κB ACTIVITY IN RAW 264.7 CELLS

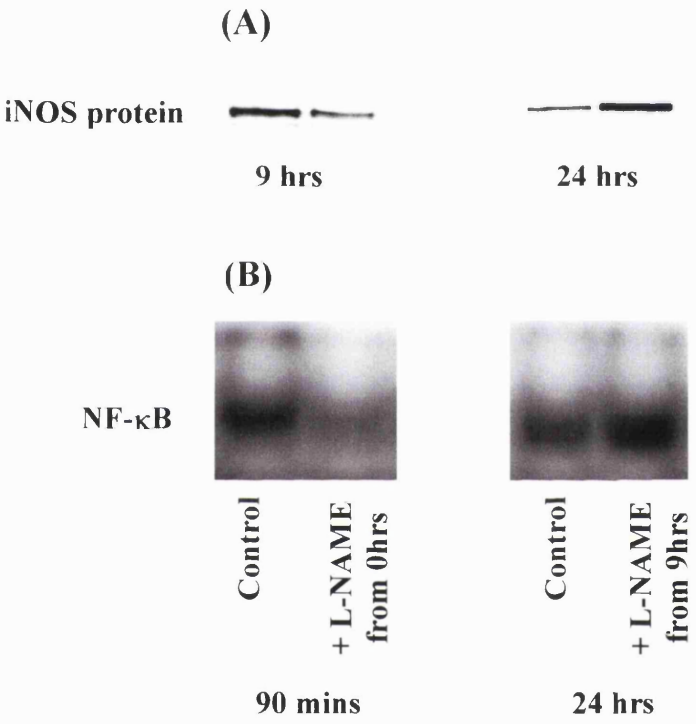


FIGURE 27. Expression of iNOS protein (A) and NF-κB activity (B) in RAW 264.7 macrophages activated with LPS (1μg/ml) in the absence and presence of L-NAME (1mM). *Left*, L-NAME added concomitantly with LPS; *right*, L-NAME added 9 hrs after activation with LPS. Protein expression was analysed by Western blot 9 hrs (*left*) and 24 hrs (*right*) after activation. NF-κB activity was assessed by EMSA 90 min (*left*) and 24 hrs (*right*) after activation. Data are representative of at least three separate experiments.

3.6.2 Effect of exogenous NO on NF-κB activity

To determine whether different concentrations of NO had divergent effects on NF-κB activity, cells were activated with LPS (1μg/ml) in the presence of increasing concentrations of the NO donor DEA-NO (30nM-300μM). At lower concentrations (30nM-3μM), DEA-NO potentiated the LPS-stimulated NF-κB activity while at higher concentrations (300μM) DEA-NO inhibited activation of NF-κB (Figure 28).

3.6.3 Effect of exogenous NO on iNOS and COX-2 expression

The activation of cells with LPS (1μg/ml) in the presence of increasing concentrations of DEA-NO (30nM-300μM) was repeated in order to elucidate whether the biphasic effect on NF-κB was reflected in pro-inflammatory protein expression.

Treatment of cells with DEA-NO (30nM-300μM) alone was unable to induce iNOS expression (Figure 29). However, the LPS-stimulated mRNA and protein expression for iNOS (Figure 30) and COX-2 (Figure 31) was potentiated at low concentrations of DEA-NO (30nM-3μM) but was inhibited at higher concentrations (300μM). This pattern mirrored the effect observed on NF-κB.

3.6.4 NO released by DEA-NO

In order to relate concentrations of free NO released to the concentration of DEA-NO used in these experiments, the peak NO release from each concentration of DEA-NO was measured using an NO electrode (Table 2). DEA-NO (30nM-300μM) produced 10-2000nM of NO.

EFFECT OF EXOGENOUS NO ON NF-κB ACTIVITY IN RAW 264.7 CELLS

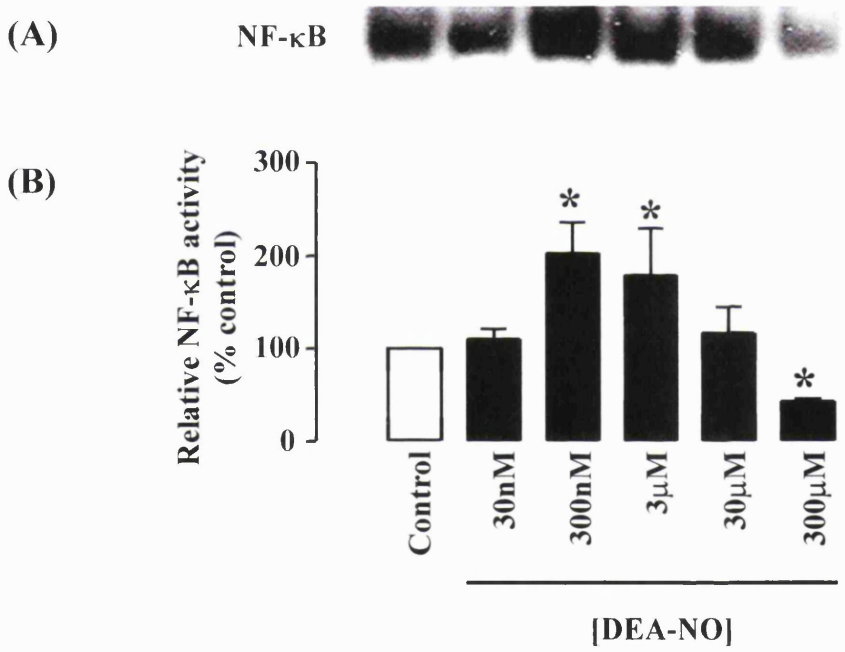


FIGURE 28. Activity of the transcription factor NF-κB in RAW 264.7 macrophages activated with LPS (1μg/ml) in the absence and presence of increasing concentrations of DEA-NO (30nM-300μM). NF-κB activity was measured by EMSA 90 mins after activation with LPS (A), and bands were analysed by densitometry (B). Data are represented as mean ± SEM density, expressed as a percentage of NF-κB activity with LPS alone ($n > 5$); * $p < 0.05$ vs control.

EFFECT OF DEA-NO ALONE ON iNOS EXPRESSION IN RAW 264.7 CELLS

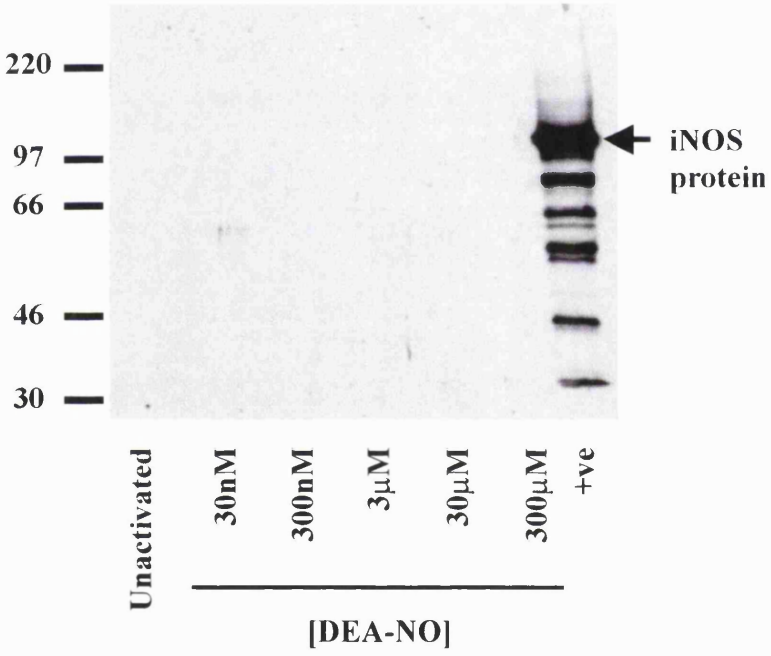


FIGURE 29. Expression of iNOS protein in RAW 264.7 macrophages incubated with increasing concentrations of DEA-NO (30nM-300μM). Expression was analysed by Western blot 24 hrs after activation. Positive control (+ve) was commercially supplied.

EFFECT OF EXOGENOUS NO ON iNOS EXPRESSION IN RAW 264.7 CELLS

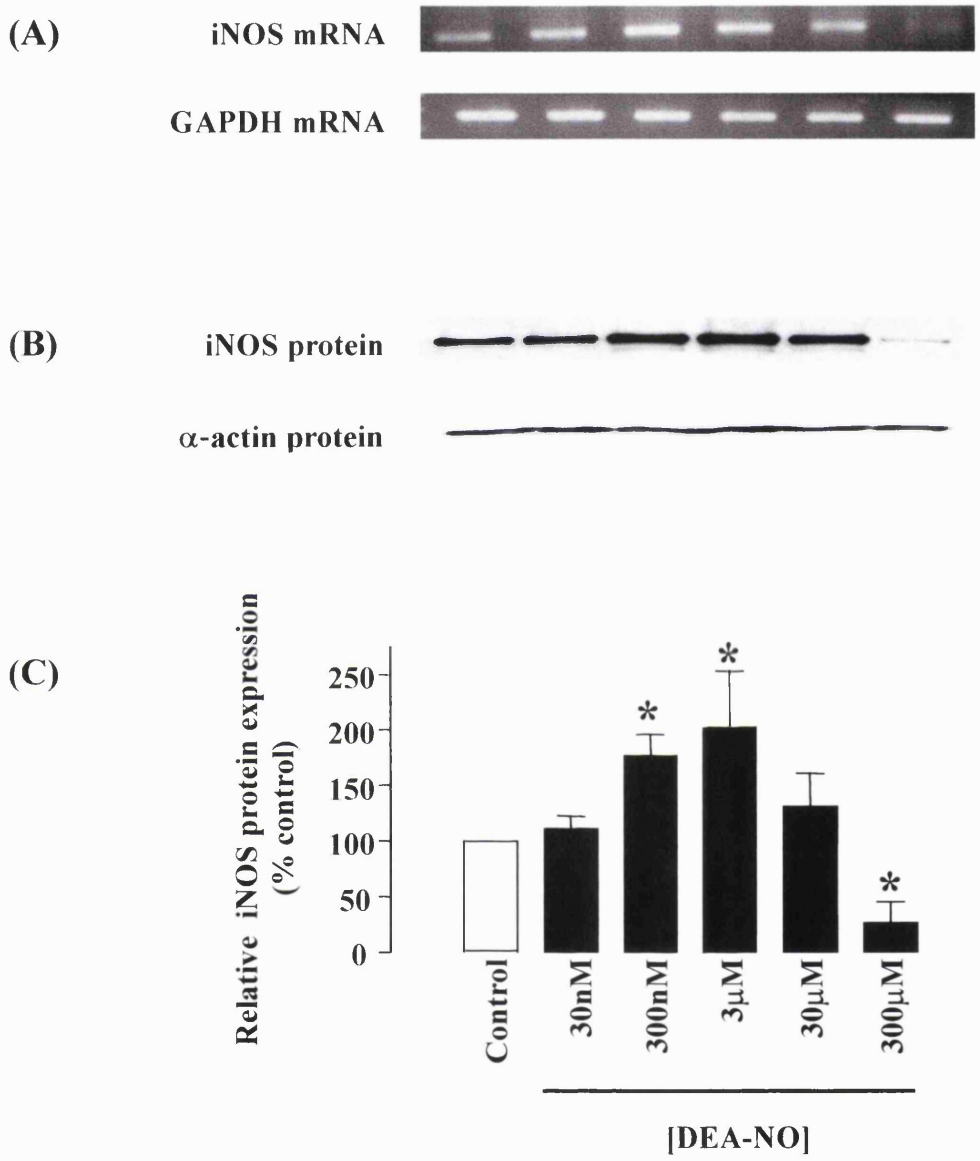


FIGURE 30. Expression of iNOS mRNA and protein in RAW 264.7 macrophages activated with LPS (1μg/ml) in the absence and presence of increasing concentrations of DEA-NO (30nM-300μM). mRNA expression was analysed by RT-PCR (A) and iNOS and α-actin protein expression were analysed by Western blot 24 hrs after activation (B). iNOS protein bands were quantified by densitometry (C) and data are represented as the mean ± SEM density, expressed as a percentage of protein expression with LPS alone ($n > 3$); * $p < 0.05$ vs control.

EFFECT OF EXOGENOUS NO ON COX-2 EXPRESSION IN RAW 264.7 CELLS

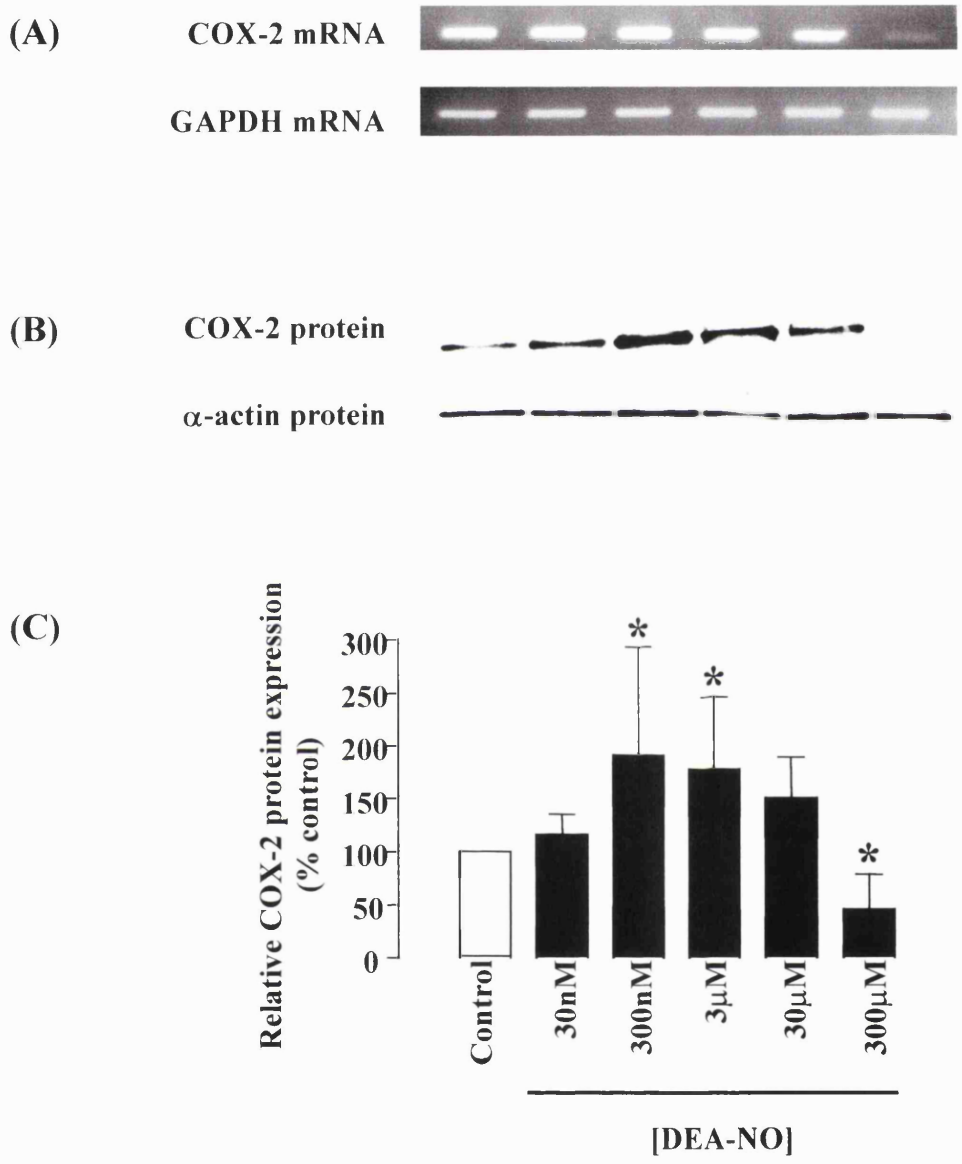


FIGURE 31. Expression of COX-2 mRNA and protein in RAW 264.7 macrophages activated with LPS (1μg/ml) in the absence and presence of increasing concentrations of DEA-NO (30nM-300μM). mRNA expression was analysed by RT-PCR (A) and COX-2 and α-actin protein expression were analysed by Western blot 24 hrs after activation (B). COX-2 protein bands were quantified by densitometry (C) and data are represented as the mean ± SEM density, expressed as a percentage of protein expression with LPS alone ($n > 3$); * $p < 0.05$ vs control.

RELEASE OF NO FROM DEA-NO

	[DEA-NO]				
	30nM	300nM	3μM	30μM	300μM
Peak [NO] (nM)	6.5 ± 1.6	21.8 ± 4.7	85.6 ± 14.8	528.9 ± 246.0	2067.8 ± 1053.9

TABLE 2. Peak [NO] produced by increasing concentrations of DEA-NO (30nM-300μM) as measured by a Clarke-type electrode in 1ml complete medium containing 10^6 RAW 264.7 cells. Data are expressed as mean ± SEM ($n \geq 4$).

3.7 ROLE OF CONSTITUTIVE NOS

3.7.1 Constitutive NOS expression in RAW 264.7 cells

Western blot analysis was conducted on whole cell extracts from RAW 264.7 cells using anti-eNOS and nNOS antibodies. nNOS expression in the RAW 264.7 cells was absent (data not shown). However, a low level of eNOS expression was observed (Figure 32).

3.7.2 Transfection of eNOS into RAW 264.7 cells

To investigate potential effects of up-regulated eNOS expression on macrophage activation in response to LPS, RAW 264.7 cells were transfected with a pcDNA3.1/eNOS plasmid. A preliminary experiment was carried out using two ratios of DNA to transfection reagent, 1:2 and 1:5, and assessing eNOS expression at different time points (24 and 48 hrs; Figure 33). The optimum results were obtained with a DNA:reagent ration of 1:2 with the transfected eNOS levels peaking after 24 hrs.

Having established a protocol with which to artificially augment eNOS expression in RAW 264.7 macrophages, cells were activated with LPS 24 hrs after transfection and iNOS expression measured after a further 24 hrs. A representative experiment in which cells were activated with LPS (1μg/ml) after transfection with eNOS or a control plasmid, where eNOS was inserted in the incorrect orientation, is shown in Figure 33. The expression of iNOS was not altered consistently by cells possessing up-regulated eNOS.

ENDOTHELIAL NOS EXPRESSION IN RAW 264.7 CELLS

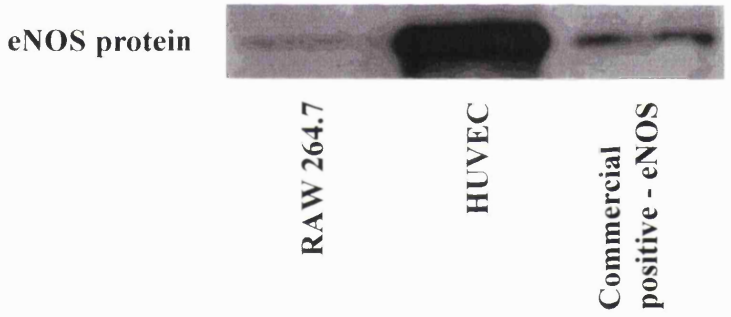


FIGURE 32. Western blot analysis of eNOS in RAW 264.7 macrophages, HUVEC and a commercially supplied positive control.

TRANSFECTION OF eNOS INTO RAW 264.7 CELLS

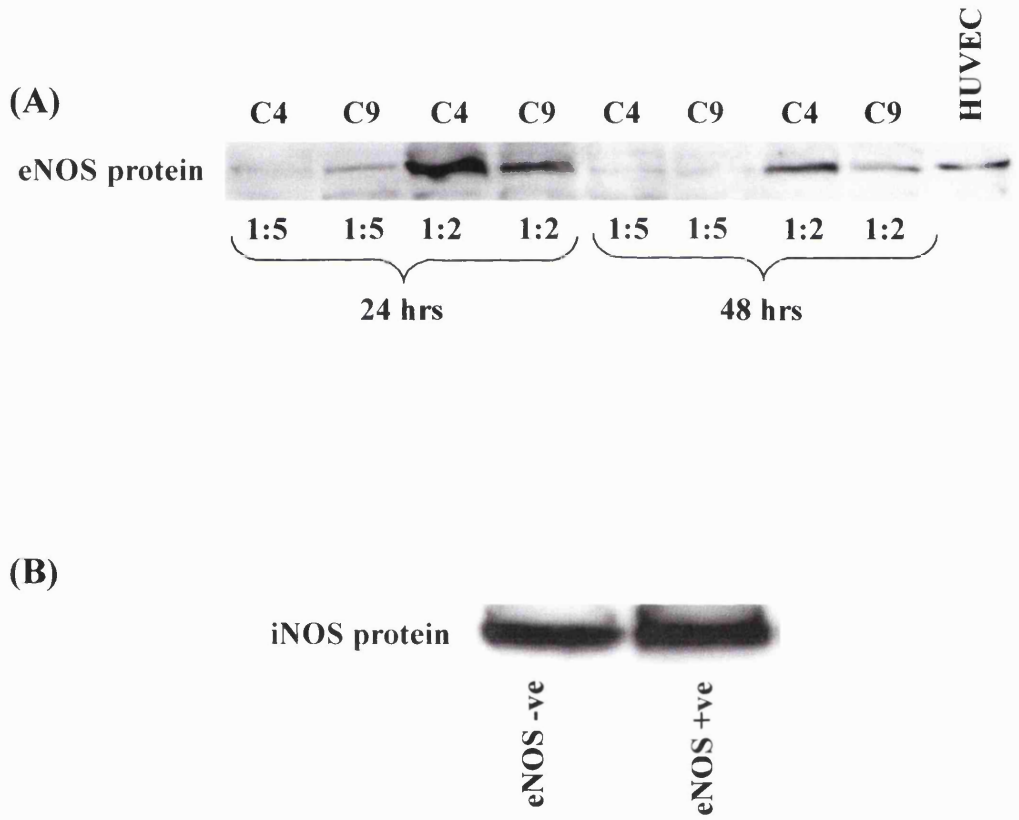


FIGURE 33. (A) Expression of eNOS protein in RAW 264.7 cells transfected with different ratios of eNOS plasmid:transfection reagent. C4 and C9 are clones which express eNOS. Expression was analysed by Western blot 24 and 48 hrs after transfection. **(B)** Expression of iNOS in RAW 264.7 cells transfected with eNOS positive or negative clones for 24 hrs before activation with LPS (1μg/ml). iNOS expression was analysed by Western blot 24 hrs after activation.

3.8 ACTIVATION OF BONE MARROW-DERIVED MACROPHAGES

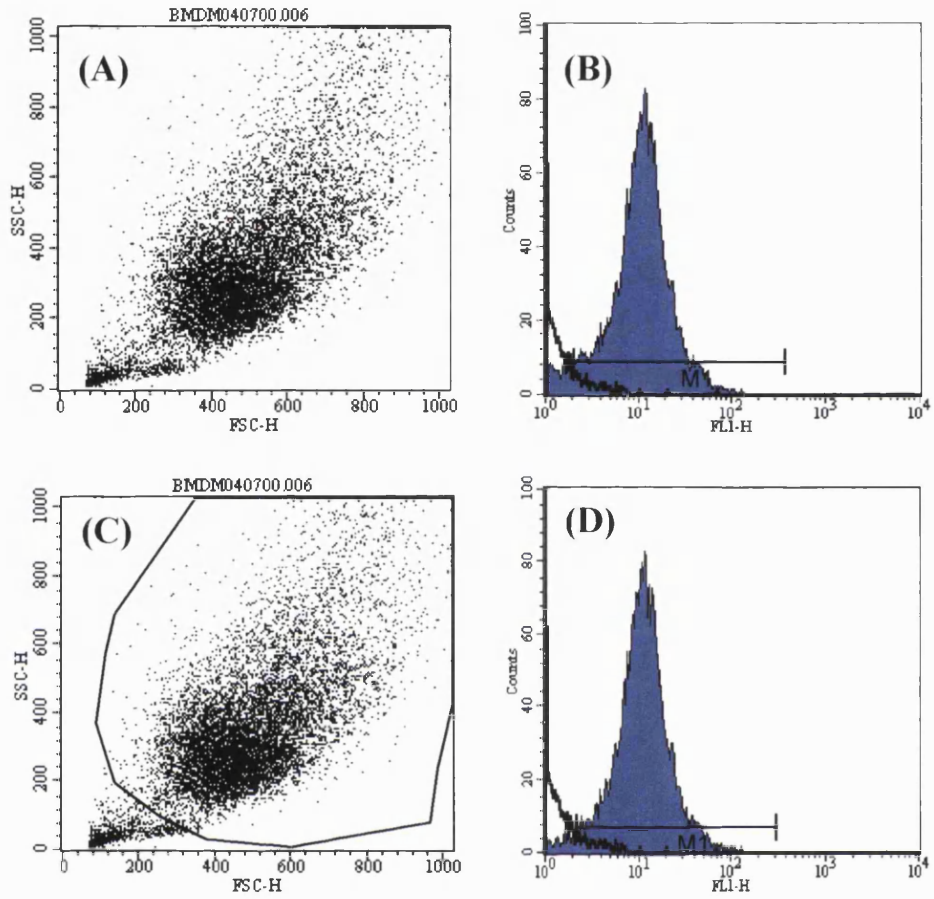
3.8.1 Characterisation of bone marrow-derived macrophages

In an attempt to obtain a more selective inhibition of distinct NOS isoforms than is possible with synthetic inhibitors, macrophages derived from eNOS and iNOS KO animals were activated with LPS and iNOS and COX expression and activity were assessed.

In order to confirm that the murine bone marrow cells had differentiated to become macrophages, FACS analysis was carried out using an antibody to the F4/80 antigen which is expressed only by murine macrophages (Hirsch *et al.*, 1981). A representative set of data obtained for eNOS WT cells is shown (Figure 34), similar results were obtained for iNOS WT and KO cells. Analysis of the major population of cells by gating and subtraction of background fluorescence showed that approximately 97% of the population of cells derived from eNOS WT and KO animals exhibited a macrophage phenotype (Figure 34). These cells were also probed with an anti-CD3 antibody, which is lymphocyte-specific, and only 1% expression was detected (Figure 35).

A Western blot was carried out in whole cell extracts from the eNOS and iNOS KO macrophages to confirm that the targeted NOS isoform was not being expressed (Figure 36). A comparison with extract from HUVEC as positive control indicated that eNOS was not expressed in KO cells. A similar level of eNOS expression was obtained in eNOS WT cells in control cells as compared to cells activated with LPS (100ng/ml) for 24 hrs suggesting LPS treatment did not alter eNOS expression. iNOS WT and KO cells were activated with LPS (100ng/ml) for 24 hrs and protein extracts were probed for iNOS expression by Western blot, with iNOS only being apparent in WT cells.

CHARACTERISATION OF BONE MARROW-DERIVED MACROPHAGES BY FLOW CYTOMETRY



(E)

Condition	% macrophages
eNOS WT (A, B)	90.64
eNOS WT (gated) (C, D)	97.63
eNOS KO	92.34
eNOS KO (gated)	97.33

FIGURE 34. Characterisation of bone marrow-derived macrophages by FACS analysis. *FSC-H*, forward scatter channel; *SSC-H*, side scatter channel; *FL1-H*, fluorescence channel 1. Distribution of eNOS WT cells without (A) and with (C) the main population of cells gated for statistical analysis. The flow cytometric profiles (B – no gating and D – with gating) show F4/80 staining (*blue*) and background staining (*black*). Line M1 allows for a subtraction of the background staining. The table (E) shows the percentage of each population which is F4/80 positive (macrophages).

CHARACTERISATION OF BONE MARROW-DERIVED MACROPHAGES BY FLOW CYTOMETRY

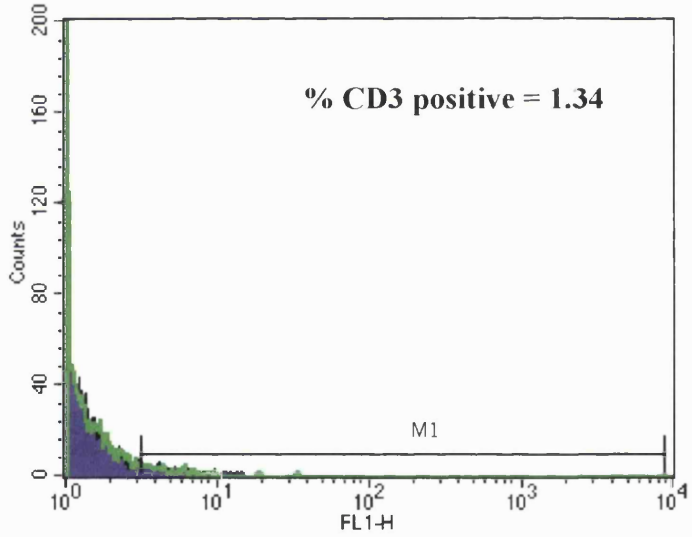


FIGURE 35. Characterisation of bone marrow-derived macrophages by FACS analysis. *FL1-H*, fluorescence channel 1. Measurement of CD3 expression in eNOS WT cells. The flow cytometric profile shows CD3 staining (*purple*) and background staining (*green*). Line M1 allows for a subtraction of the background staining.

CHARACTERISATION OF eNOS AND iNOS WT AND KO CELLS BY WESTERN BLOT

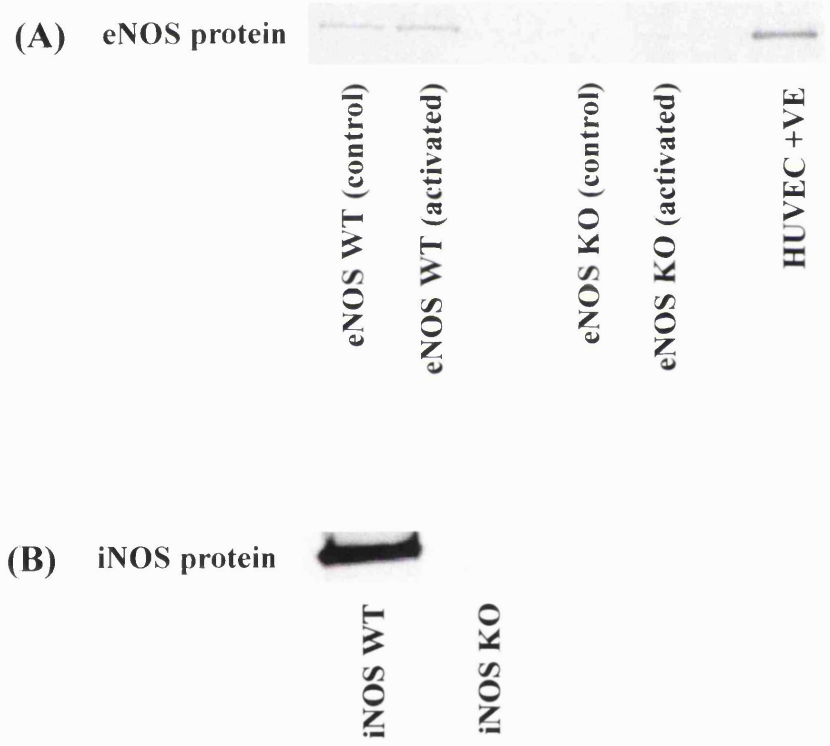


FIGURE 36. Western blot for eNOS (A) and iNOS (B) in cells derived from eNOS (A) and iNOS (B) WT and KO mice. Cells were treated with or without LPS (100ng/ml) for 24 hrs. Data are representative of at least four separate experiments.

3.8.2 Activation of eNOS WT and KO bone marrow-derived macrophages with LPS

Murine bone marrow-derived macrophages cultured from eNOS WT and KO animals were activated with LPS and iNOS and COX expression and activity were measured (Figures 37-42). A concentration of 100ng/ml LPS was selected as this gave maximal protein expression (Figure 37). A profile of iNOS and COX-2 expression similar to that of the RAW 264.7 cells was observed with protein levels increasing from 6 hrs, peaking between 12 and 24 hrs and then falling at 48 hrs. Similar concentrations of nitrite and PGE_2 were also observed.

While the time course of expression remained unchanged, the levels of iNOS expression and activity were reduced in eNOS KO cells at all time points (Figures 38 and 39) with approximately 50% reduction in peak protein expression and nitrite production.

In contrast, the deletion of eNOS led to an increase in both COX-2 expression and PGE_2 production at each point up to 24 hrs, with approximately 30% higher peak protein expression levels in eNOS KO and a 50% increase in PGE_2 concentration (Figures 40 and 41). At 48 hrs COX-2 expression reached equivalent levels in both WT and KO cells (Figure 40).

COX-1 expression was equivalent in eNOS WT and KO cells following LPS activation (Figure 42).

CONCENTRATION RESPONSE TO LPS IN eNOS WT BONE MARROW-DERIVED MACROPHAGES

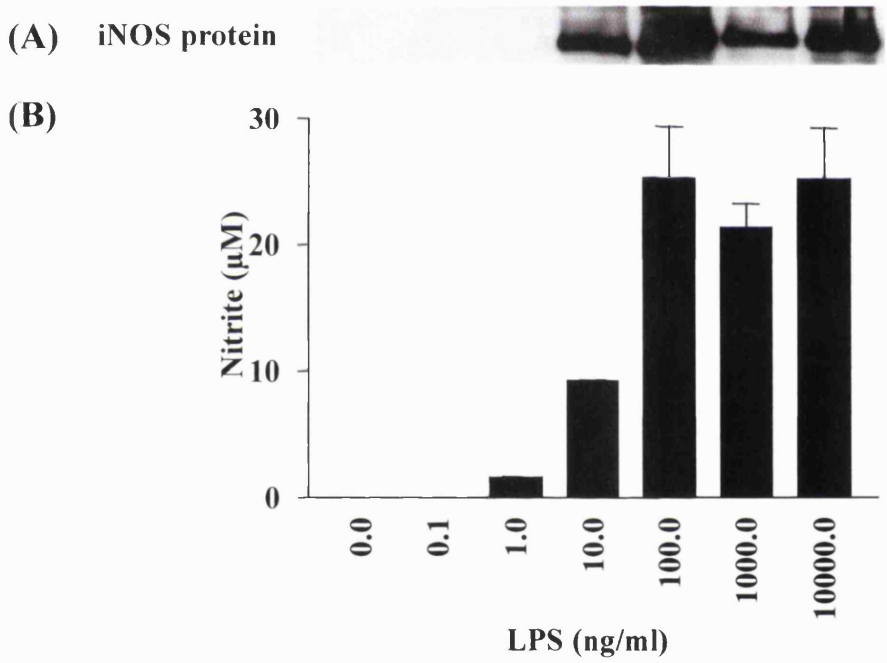


FIGURE 37. Expression of iNOS protein and nitrite production in eNOS WT bone marrow-derived macrophages activated with increasing concentrations of LPS (0-10,000ng/ml). Protein expression was analysed by Western blot (A), and nitrite concentrations measured by Griess reaction (B) after 24 hours. Data are represented as mean ± SEM nitrite production under each condition (n = 3).

iNOS EXPRESSION IN eNOS WT AND KO BONE MARROW-DERIVED MACROPHAGES ACTIVATED WITH LPS

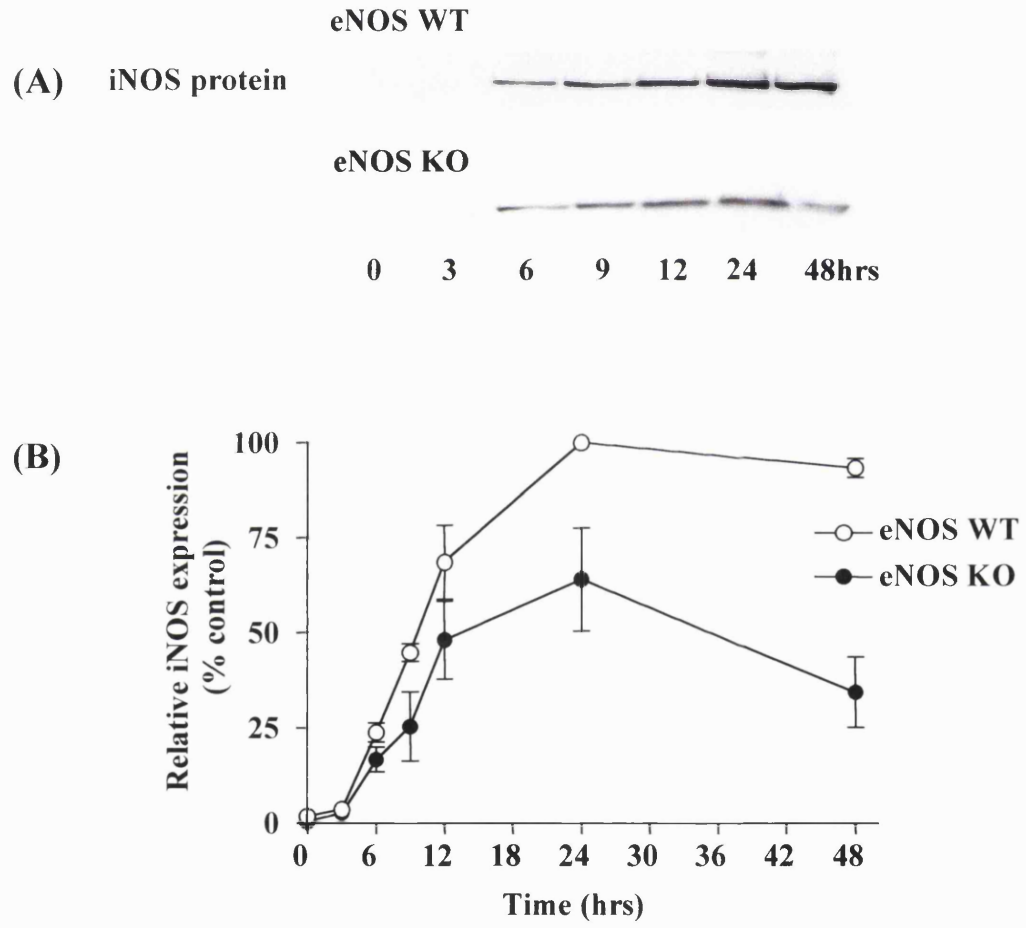


FIGURE 38. Expression of iNOS protein in eNOS WT and KO bone marrow-derived macrophages activated with LPS (100ng/ml). Protein expression was analysed by Western blot (A) and bands were quantified by densitometry (B). Data are represented as the mean ± SEM density, expressed as a percentage of peak protein expression in WT cells (n = 5).

iNOS ACTIVITY IN eNOS WT AND KO BONE MARROW-DERIVED MACROPHAGES ACTIVATED WITH LPS

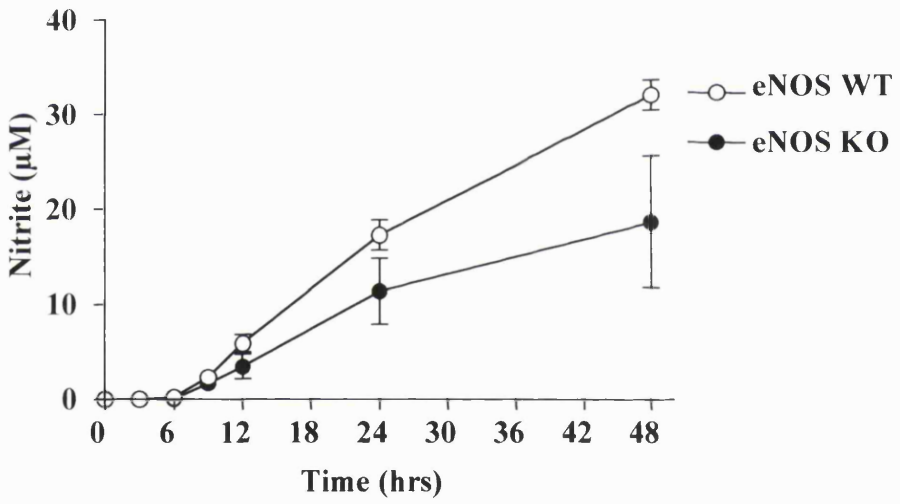


FIGURE 39. Accumulation of nitrite in the culture medium of eNOS WT and KO bone marrow-derived macrophages activated with LPS (100ng/ml). Nitrite was measured by Griess reaction. Data are represented as the mean ± SEM nitrite production under each condition (n = 5).

COX-2 EXPRESSION IN eNOS WT AND KO BONE MARROW-DERIVED MACROPHAGES ACTIVATED WITH LPS

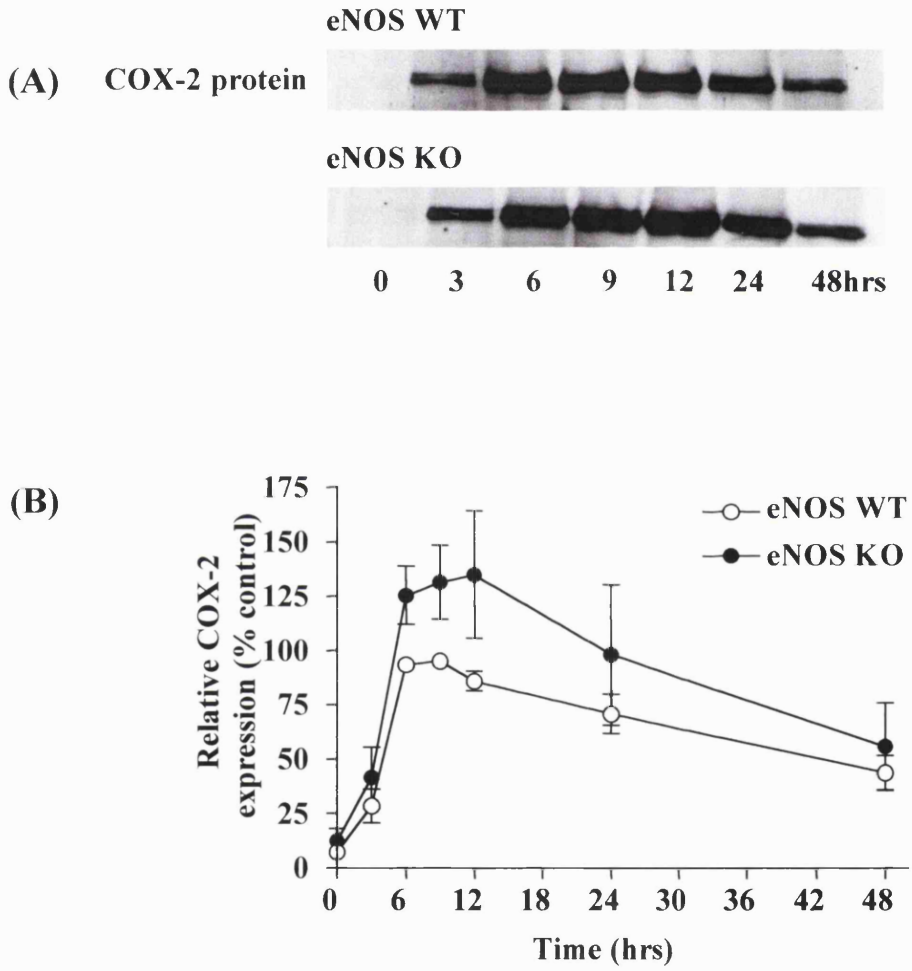


FIGURE 40. Expression of COX-2 protein in eNOS WT and KO bone marrow-derived macrophages activated with LPS (100ng/ml). Protein expression was analysed by Western blot (A) and bands were quantified by densitometry (B). Data are represented as the mean ± SEM density, expressed as a percentage of peak protein expression in WT cells (n = 5).

COX-2 ACTIVITY IN eNOS WT AND KO BONE MARROW-DERIVED MACROPHAGES ACTIVATED WITH LPS

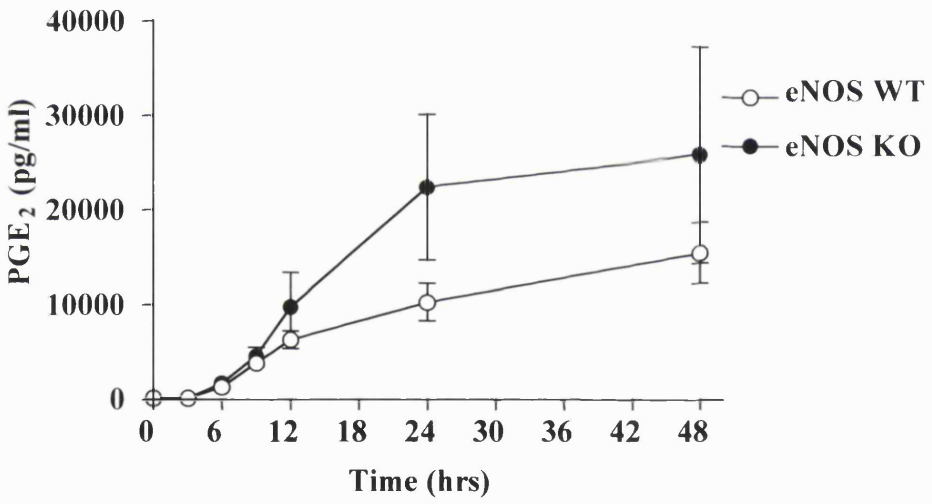


FIGURE 41. Accumulation of PGE_2 in the culture medium of eNOS WT and KO bone marrow-derived macrophages activated with LPS (100ng/ml). PGE_2 was measured by ELISA. Data are represented as the mean ± SEM PGE_2 production under each condition (n = 5).

COX-1 EXPRESSION IN eNOS WT AND KO BONE MARROW-DERIVED MACROPHAGES ACTIVATED WITH LPS

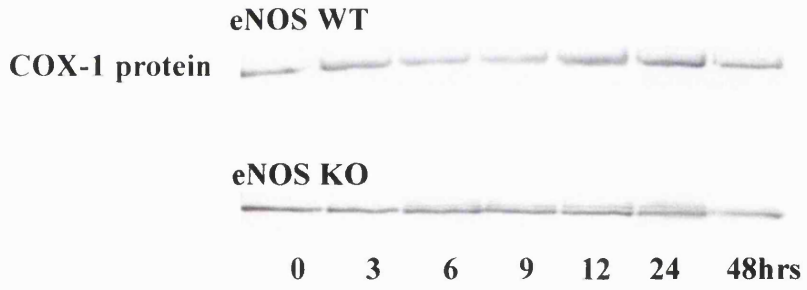


FIGURE 42. Expression of COX-1 protein in eNOS WT and KO bone marrow-derived macrophages activated with LPS (100ng/ml). Protein expression was analysed by Western blot. Data are representative of five separate experiments.

3.8.3 Activation of iNOS WT and KO bone marrow-derived macrophages with LPS

A more rapid pattern of COX-2 expression was observed in the iNOS WT and KO cells than in eNOS WT and KO or RAW 264.7 cells following LPS (100ng/ml) activation. COX-2 levels peaked after 9 hrs and then fell to almost basal levels after 48 hrs. The iNOS WT cells produced a lower concentration of nitrite than the other cells studied while nitrite production was not apparent in KO cells (Figures 43 and 44).

Since no iNOS expression and associated NO production is observed in the iNOS KO cells, COX expression and activity was followed over 48 hrs. While at earlier time points (up to 9 hrs) COX-2 protein expression was unaffected by iNOS deletion, at later time points (12 to 24 hrs) there was a small reduction in COX-2 expression in KO cells of about 10%. At 48 hrs this difference was no longer apparent, with expression falling to basal levels in both WT and KO cells (Figure 44). At time points from 12 hrs a reduced production of PGE_2 was apparent in the iNOS KO cells, with peak PGE_2 concentration in KO cells approximately 25% lower than in WT (Figure 45).

COX-1 expression was equivalent in iNOS WT and KO cells following LPS activation (Figure 46).

NITRITE PRODUCTION IN iNOS WT AND KO BONE MARROW-DERIVED MACROPHAGES ACTIVATED WITH LPS

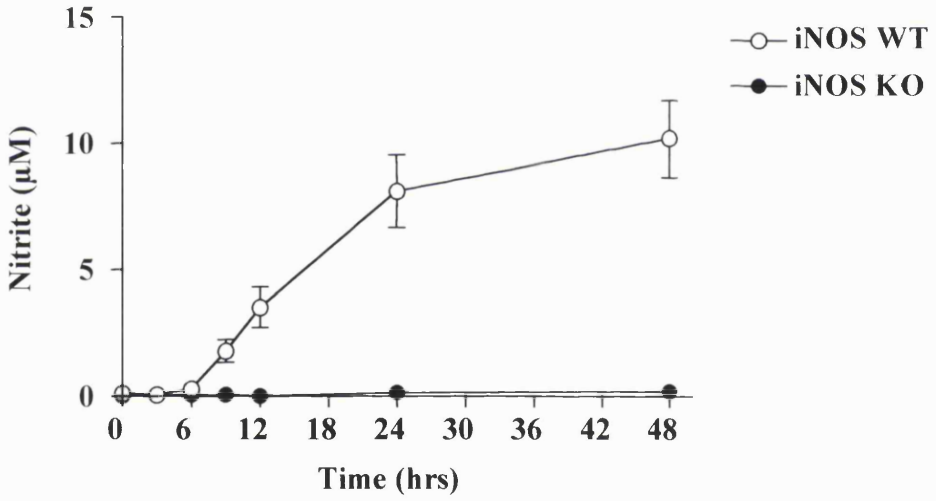


FIGURE 43. Accumulation of nitrite in the culture medium of iNOS WT and KO bone marrow-derived macrophages activated with LPS (100ng/ml). Nitrite was measured by Griess reaction. Data are represented as the mean ± SEM nitrite production under each condition (n = 4).

COX-2 EXPRESSION IN iNOS WT AND KO BONE MARROW-DERIVED MACROPHAGES ACTIVATED WITH LPS

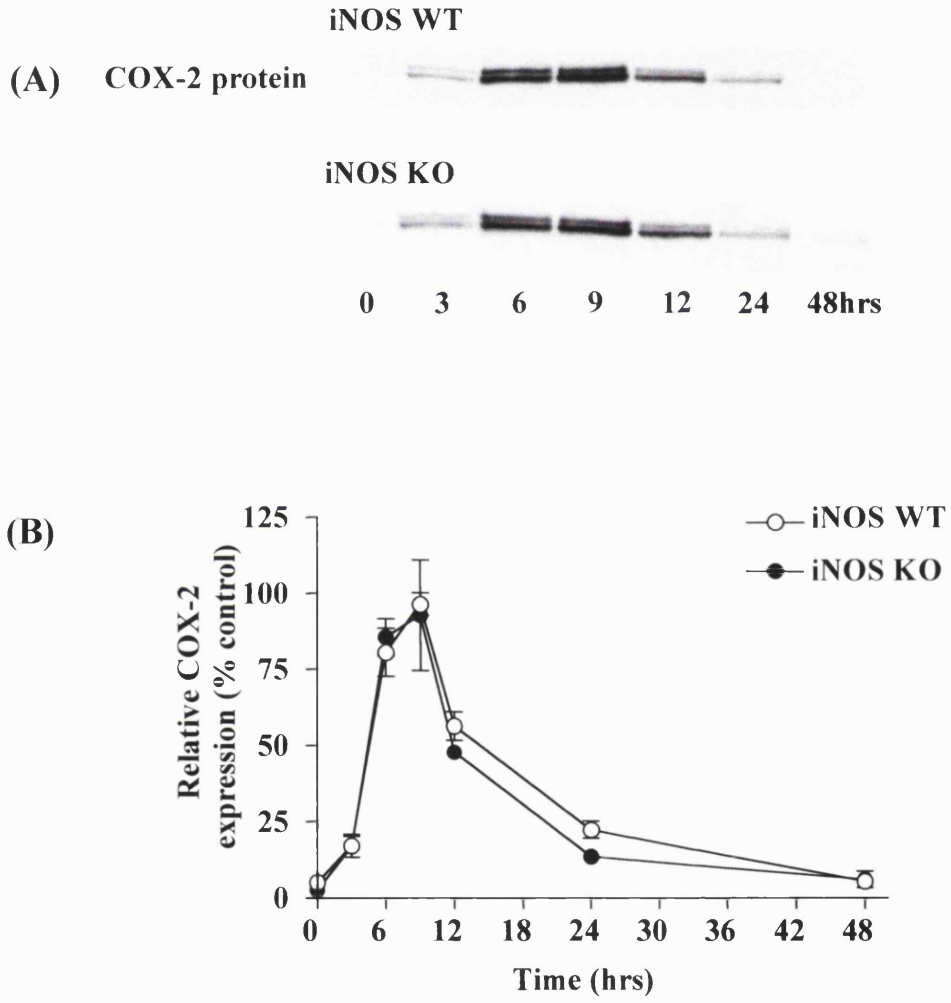


FIGURE 44. Expression of COX-2 protein in iNOS WT and KO bone marrow-derived macrophages activated with LPS (100ng/ml). Protein expression was analysed by Western blot (A) and bands were quantified by densitometry (B). Data are represented as the mean ± SEM density, expressed as a percentage of peak protein expression in WT cells (n = 4).

COX-2 ACTIVITY IN iNOS WT AND KO BONE MARROW-DERIVED MACROPHAGES ACTIVATED WITH LPS

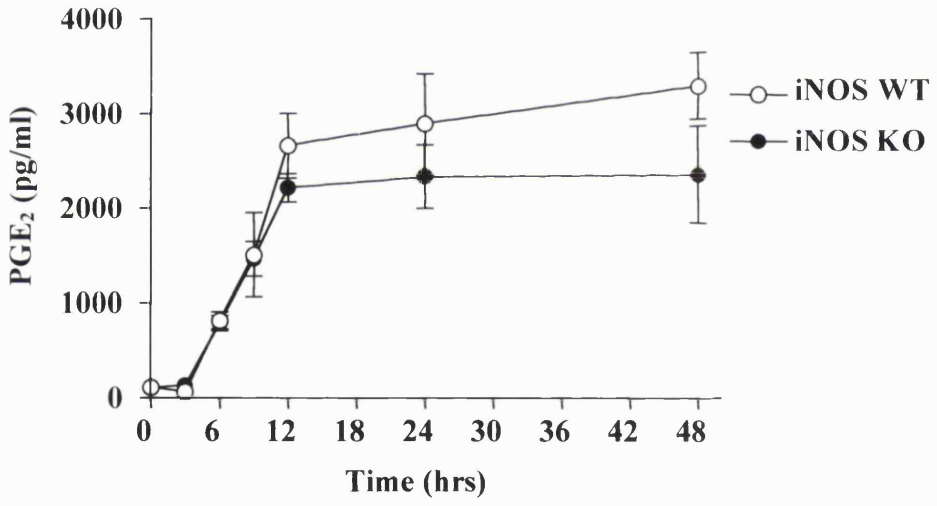


FIGURE 45. Accumulation of PGE_2 in the culture medium of iNOS WT and KO bone marrow-derived macrophages activated with LPS (100ng/ml). PGE_2 was measured by ELISA. Data are represented as the mean ± SEM PGE_2 production under each condition (n = 4).

COX-1 EXPRESSION IN iNOS WT AND KO BONE MARROW-DERIVED MACROPHAGES ACTIVATED WITH LPS

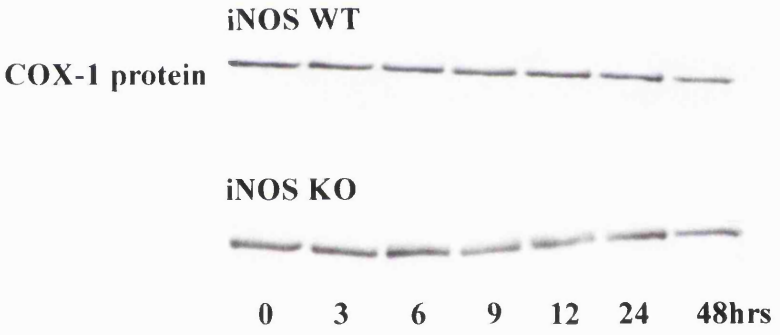


FIGURE 46. Expression of COX-1 protein in iNOS WT and KO bone marrow-derived macrophages activated with LPS (100ng/ml). Protein expression was analysed by Western blot. Data are representative of four separate experiments.

3.9 EFFECT OF cGMP ON iNOS AND COX-2 EXPRESSION IN BONE MARROW-DERIVED MACROPHAGES

3.9.1 Effect of the sGC activator, BAY 41-2272

In order to ascertain whether cGMP had a role in the effects of NO on macrophage activation, bone marrow-derived macrophages prepared from eNOS WT animals were pre-incubated with increasing concentrations of the sGC activator, BAY 41-2272 (0–10μM). The cells were then activated with 100ng/ml LPS and iNOS and COX expression were monitored after 9 and 24 hrs (chosen to represent early and later time points of activation).

The sGC activator, BAY 41-2272, led to a concentration-dependent potentiation of LPS-stimulated iNOS expression after both 9 and 24 hrs activation, with 10μM BAY 41-2272 increasing iNOS expression by ~50% (Figure 47). The lower concentrations of the sGC activator did not have a significant effect on iNOS activity but nitrite production was increased by the highest concentration (10μM; Figure 48).

A similar concentration-dependent potentiation of LPS-activated COX-2 expression was observed with BAY 41-2272, after 9 and 24 hrs (Figure 49). However, a concentration-dependent decrease in PGE_2 production was apparent at both time points (Figure 50).

COX-1 expression was not affected at any concentration of BAY 41-2272 (Figure 51).

EFFECT OF THE sGC ACTIVATOR, BAY 41-2272, ON iNOS EXPRESSION IN BONE MARROW-DERIVED MACROPHAGES

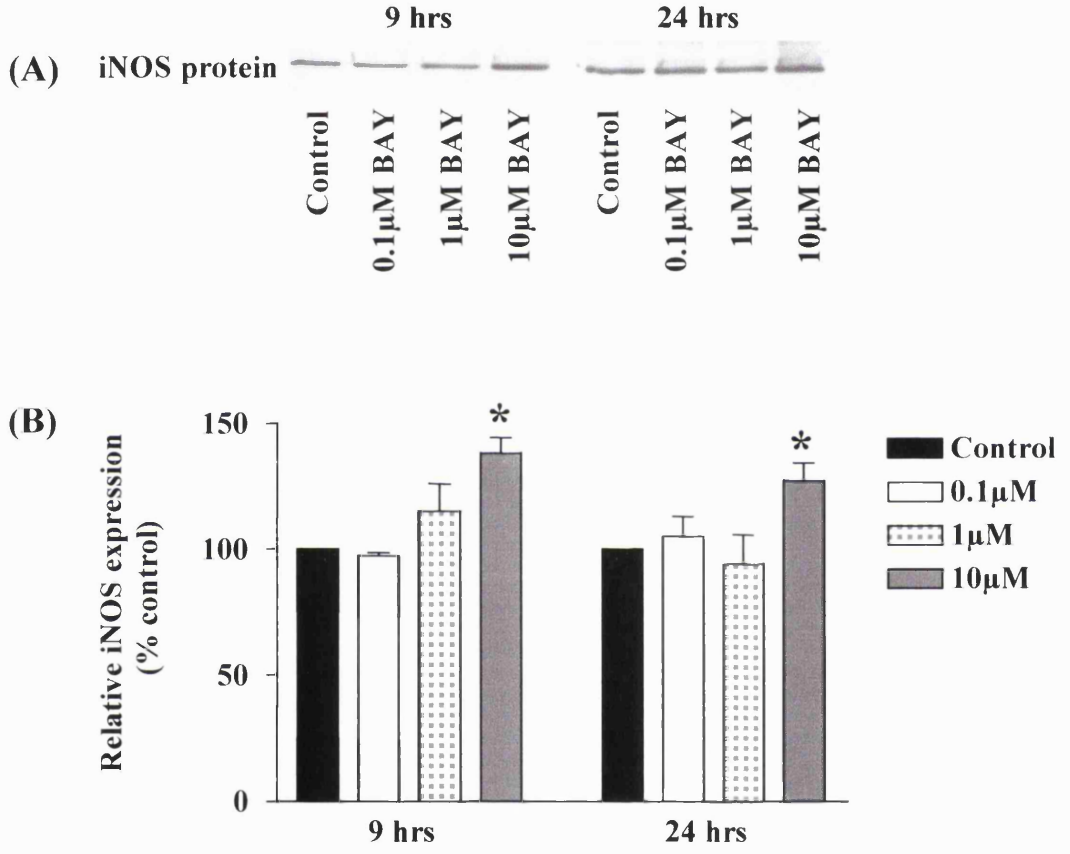


FIGURE 47. iNOS protein expression in eNOS WT bone marrow-derived macrophages activated with LPS (100ng/ml) in the absence and presence of increasing concentrations of BAY 41-2272 (0.1-10μM). Protein expression was analysed by Western blot (A) and bands were quantified by densitometry (B). Data are represented as the mean ± SEM density, expressed as a percentage of protein expression with LPS alone (n = 3); * $p < 0.05$ vs control.

EFFECT OF THE sGC ACTIVATOR, BAY 41-2272, ON iNOS ACTIVITY IN BONE MARROW-DERIVED MACROPHAGES

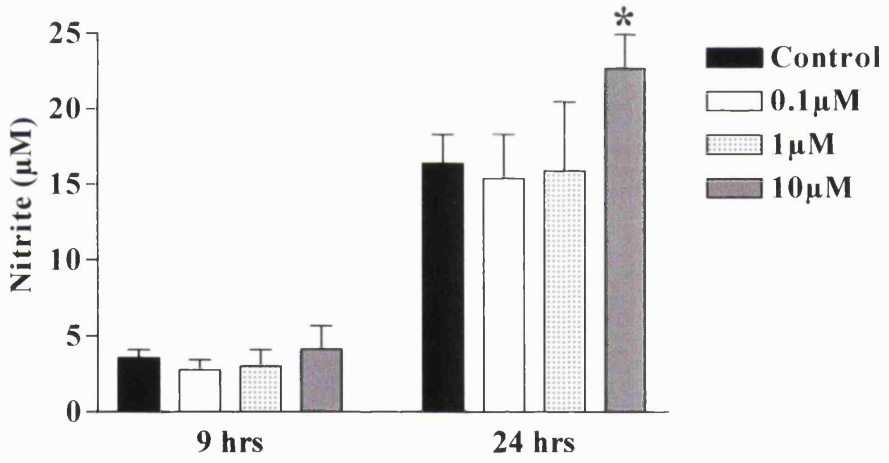


FIGURE 48. Accumulation of nitrite in eNOS WT bone marrow-derived macrophages activated with LPS (100ng/ml) in the absence and presence of increasing concentrations of BAY 41-2272 (0.1-10μM). Nitrite was measured by Griess reaction. Data are represented as the mean ± SEM nitrite production under each condition (n = 3); * $p < 0.05$ vs control.

EFFECT OF THE sGC ACTIVATOR, BAY 41-2272, ON COX-2 EXPRESSION IN BONE MARROW-DERIVED MACROPHAGES

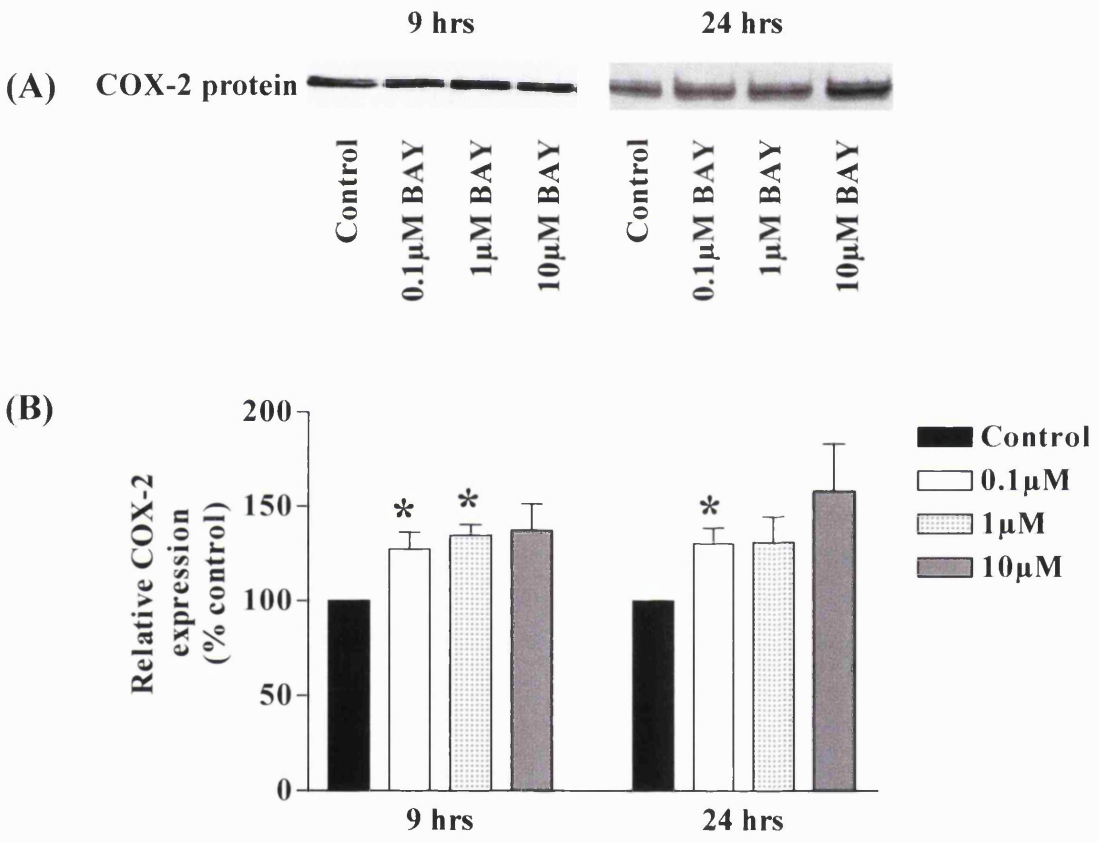


FIGURE 49. Expression of COX-2 protein in eNOS WT bone marrow-derived macrophages activated with LPS (100ng/ml) in the absence and presence of increasing concentrations of BAY 41-2272 (0.1-10μM). Protein expression was analysed by Western blot (A) and bands were quantified by densitometry (B). Data are represented as the mean ± SEM density, expressed as a percentage of protein expression with LPS alone (n = 3); * $p < 0.05$ vs control.

EFFECT OF THE sGC ACTIVATOR, BAY 41-2272, ON COX-2 ACTIVITY IN BONE MARROW-DERIVED MACROPHAGES

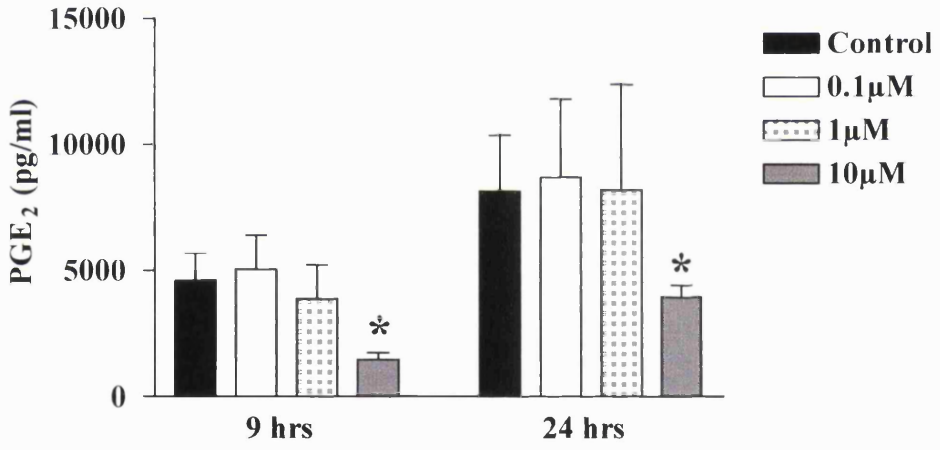


FIGURE 50. Accumulation of PGE_2 in the culture medium of eNOS WT bone marrow-derived macrophages activated with LPS (100ng/ml) in the absence and presence of increasing concentrations of BAY 41-2272 (0.1-10μM). PGE_2 was measured by ELISA. Data are represented as the mean ± SEM PGE_2 production under each condition (n = 3); * $p < 0.05$ vs control.

EFFECT OF THE sGC ACTIVATOR, BAY 41-2272, ON COX-1 EXPRESSION IN BONE MARROW-DERIVED MACROPHAGES

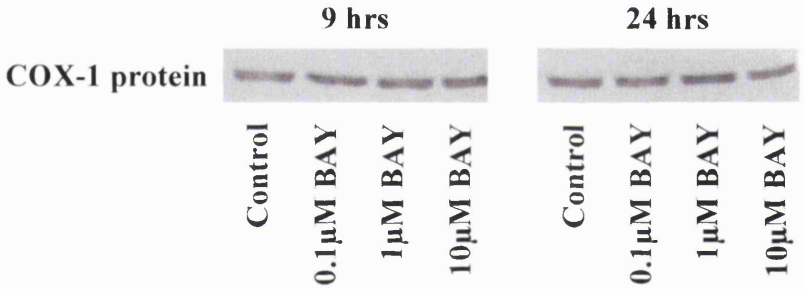


FIGURE 51. Expression of COX-1 protein in eNOS WT bone marrow-derived macrophages activated with LPS (100ng/ml) in the absence and presence of increasing concentrations of BAY 41-2272 (0.1-10μM). Protein expression was analysed by Western blot. Data are representative of three separate experiments.

3.9.2 Effect of the sGC inhibitor, ODQ

In order to substantiate the observed role of sGC/cGMP in macrophage activation, the effect of the sGC inhibitor, ODQ (10μM), on LPS-stimulated iNOS expression and activity was studied in eNOS WT bone marrow-derived macrophages. This concentration of ODQ has been shown to be effective in previous studies with cultured cells (Berkels *et al.*, 2000; Ziolo *et al.*, 2001).

The presence of ODQ led to a reduction in LPS-stimulated iNOS expression and nitrite production at both 9 and 24 hrs after activation. ODQ also reversed the stimulatory effect that BAY 41-2272 had on iNOS expression and activity (Figures 52 and 53).

EFFECT OF THE sGC INHIBITOR, ODQ, ON iNOS EXPRESSION IN THE PRESENCE AND ABSENCE OF BAY 41-2272 IN BONE MARROW-DERIVED MACROPHAGES

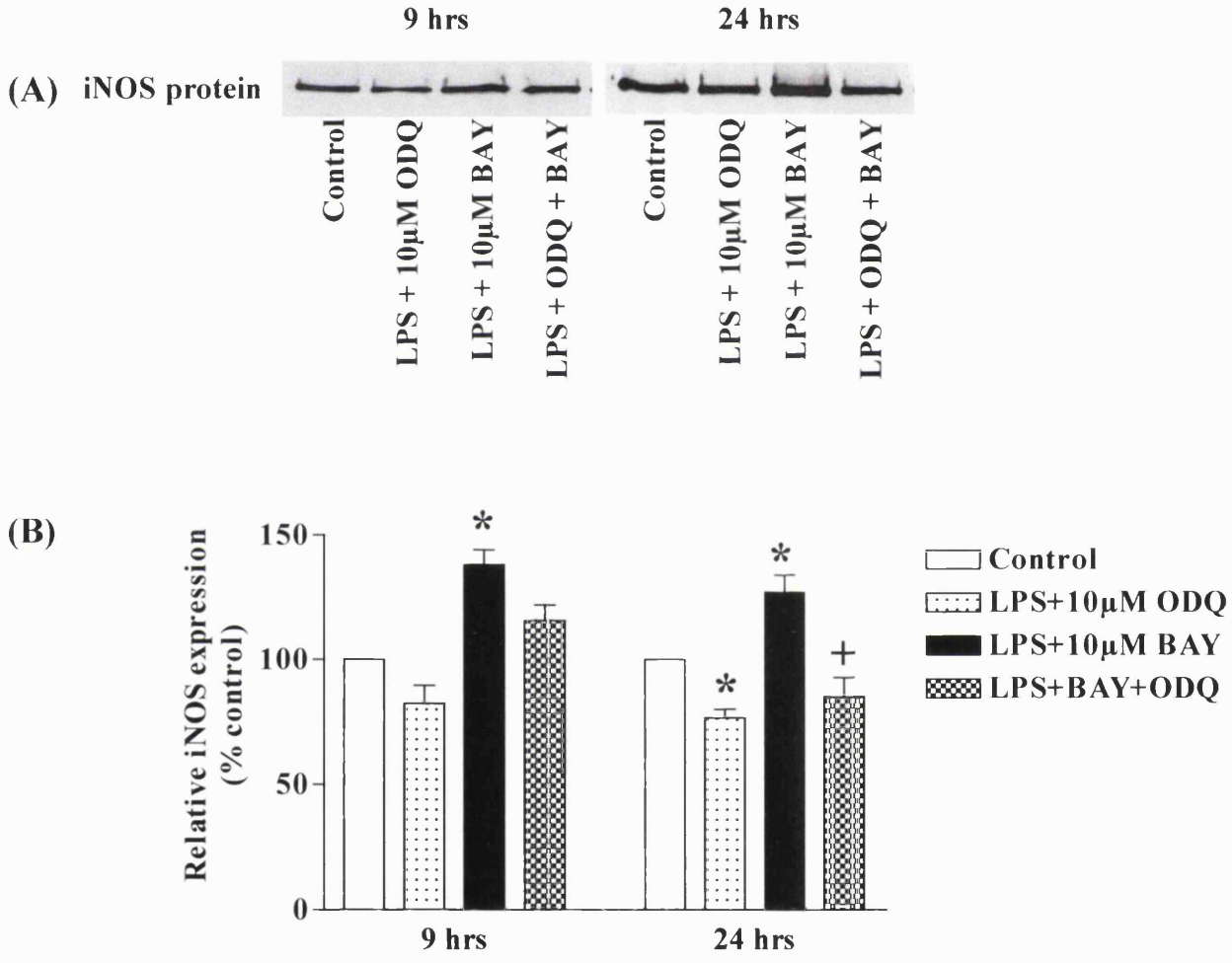


FIGURE 52. Expression of iNOS protein in eNOS WT bone marrow-derived macrophages activated with LPS (100ng/ml) in the absence and presence of ODQ (10μM) and/or BAY 41-2272 (10μM). Protein expression was analysed by Western blot (A) and bands were quantified by densitometry (B). Data are represented as the mean ± SEM density, expressed as a percentage of protein expression with LPS alone (n = 3); * $p < 0.05$ vs control; + $p < 0.05$ vs LPS + 10μM BAY 41-2272.

EFFECT OF THE sGC INHIBITOR, ODQ, ON iNOS ACTIVITY IN THE PRESENCE AND ABSENCE OF BAY 41-2272 IN BONE MARROW-DERIVED MACROPHAGES

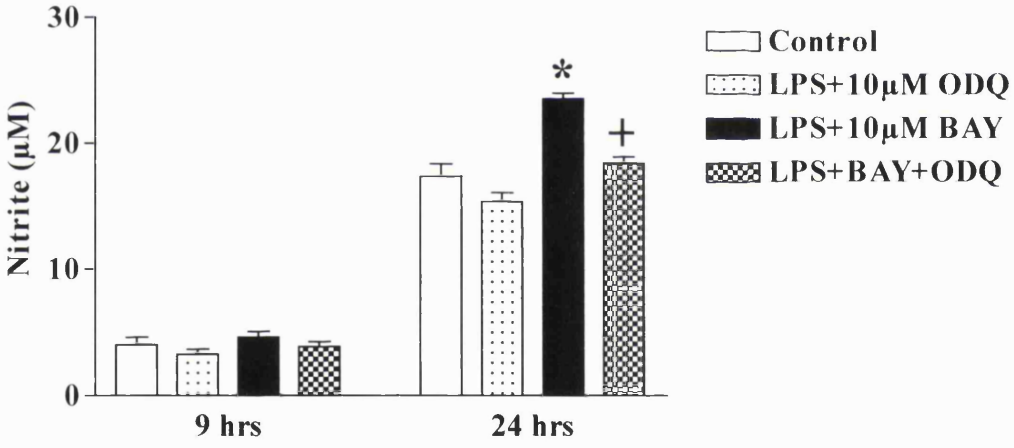


FIGURE 53. Accumulation of nitrite in the culture medium of eNOS WT bone marrow-derived macrophages activated with LPS (100ng/ml) in the absence and presence of ODQ (10μM) and/or BAY 41-2272 (10μM). Nitrite was measured by Griess reaction. Data are represented as the mean ± SEM nitrite production under each condition (n = 3); * $p < 0.05$ vs control; + $p < 0.05$ vs LPS + 10μM BAY 41-2272.

3.10 EFFECT OF THE ADENYLATE CYCLASE ACTIVATOR, FORSKOLIN, ON iNOS AND COX EXPRESSION AND ACTIVITY IN BONE MARROW-DERIVED MACROPHAGES

In order to ascertain whether the sibling cyclic nucleotide, cAMP, also had a role in macrophage activation, bone marrow-derived macrophages extracted from eNOS WT animals were pre-incubated with increasing concentrations of the adenylate cyclase activator, forskolin (0–50μM). After 9 hrs iNOS expression was unaffected by any of the concentrations of forskolin (Figure 54). At this time point, nitrite production was also unaffected (Figure 55). However, after 24 hrs, forskolin led to a concentration-dependent decrease in LPS-induced iNOS expression and activity (Figures 54 and 55).

At 9 hrs, the lower concentrations of forskolin (0.1 and 1μM) potentiated the LPS-induced COX-2 expression while the highest concentration (50μM) inhibited COX-2 expression (Figure 56). After 24 hrs forskolin produced a concentration-dependent decrease in COX-2 expression; these inhibitory effects were mirrored by changes in PGE_2 production at both time points (Figures 56 and 57).

COX-1 was not affected by any concentration of forskolin at any time point (Figure 58).

EFFECT OF THE ADENYLATE CYCLASE ACTIVATOR, FORSKOLIN, ON iNOS EXPRESSION IN BONE MARROW-DERIVED MACROPHAGES

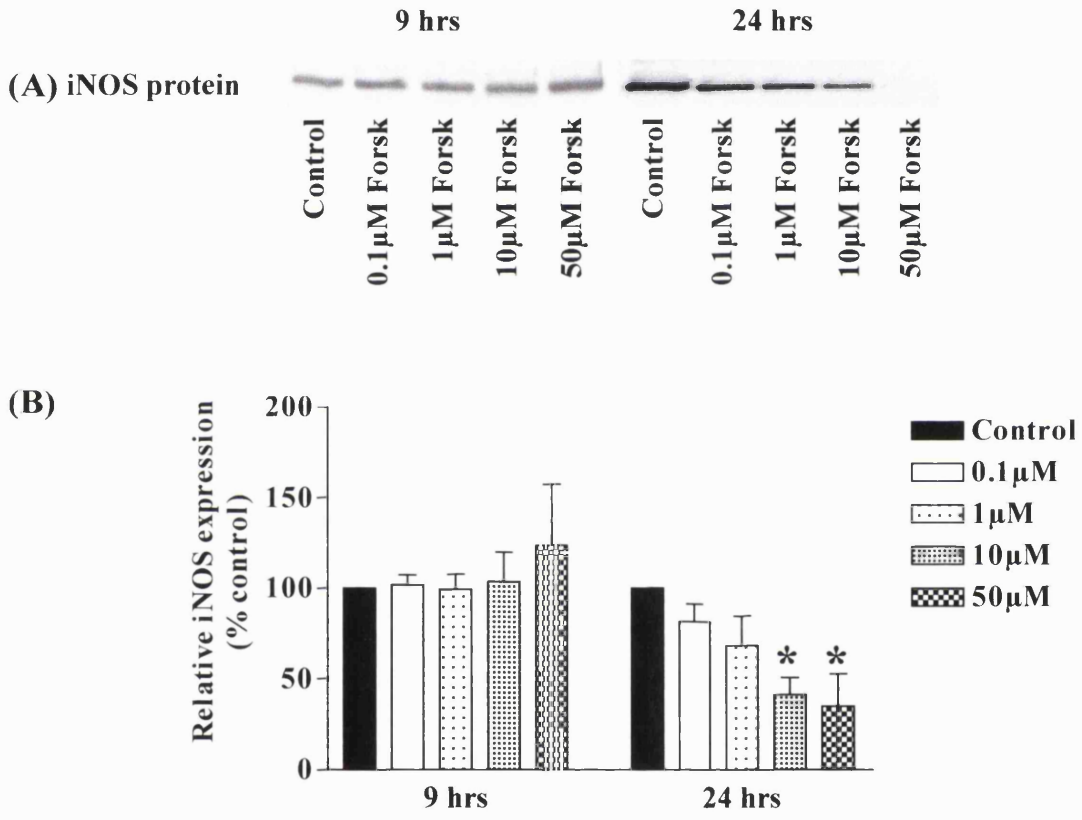


FIGURE 54. Expression of iNOS protein in eNOS WT bone marrow-derived macrophages activated with LPS (100ng/ml) in the absence and presence of increasing concentrations of forskolin (0.1-50μM). Protein expression was analysed by Western blot (A) and bands were quantified by densitometry (B). Data are represented as the mean ± SEM density, expressed as a percentage of protein expression with LPS alone (n = 3); * $p < 0.05$ vs control.

EFFECT OF THE ADENYLATE CYCLASE ACTIVATOR, FORSKOLIN, ON iNOS ACTIVITY IN BONE MARROW-DERIVED MACROPHAGES

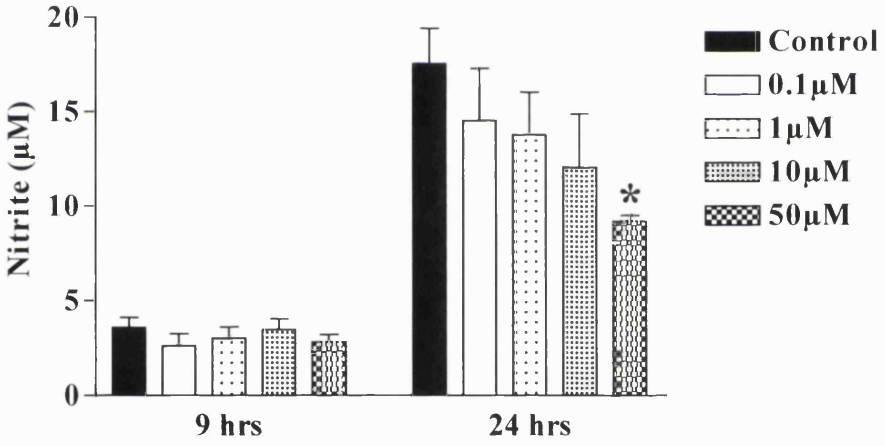


FIGURE 55. Accumulation of nitrite in the culture medium of eNOS WT bone marrow-derived macrophages activated with LPS (100ng/ml) in the absence and presence of increasing concentrations of forskolin (0.1-50μM). Nitrite was measured by Griess reaction. Data are represented as the mean ± SEM nitrite production under each condition (n = 3); * $p < 0.05$ vs control.

EFFECT OF THE ADENYLATE CYCLASE ACTIVATOR, FORSKOLIN, ON COX-2 EXPRESSION IN BONE MARROW-DERIVED MACROPHAGES

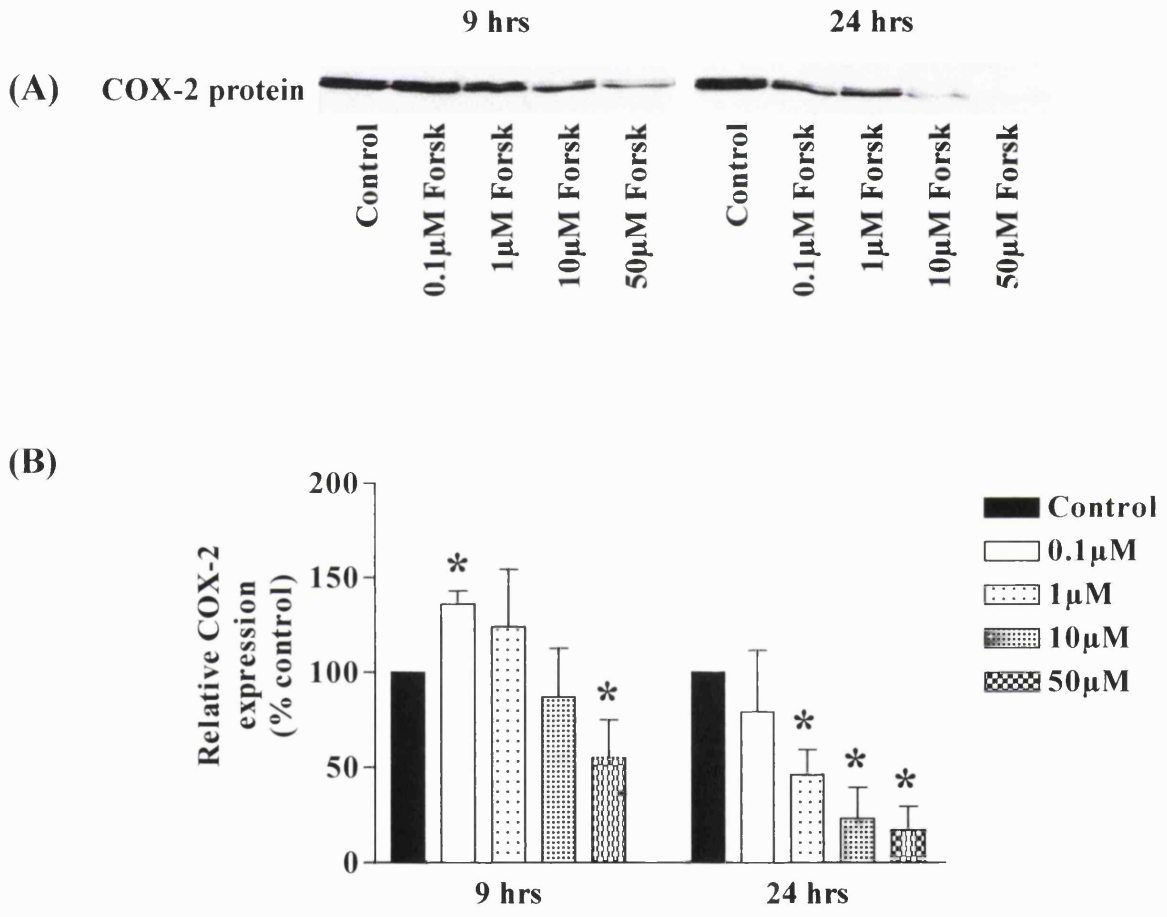


FIGURE 56. Expression of COX-2 protein in eNOS WT bone marrow-derived macrophages activated with LPS (100ng/ml) in the absence and presence of increasing concentrations of forskolin (0.1-50μM). Protein expression was analysed by Western blot (A) and bands were quantified by densitometry (B). Data are represented as the mean ± SEM density, expressed as a percentage of protein expression with LPS alone (n = 3); * $p < 0.05$ vs control.

EFFECT OF THE ADENYLATE CYCLASE ACTIVATOR, FORSKOLIN, ON COX-2 ACTIVITY IN BONE MARROW-DERIVED MACROPHAGES

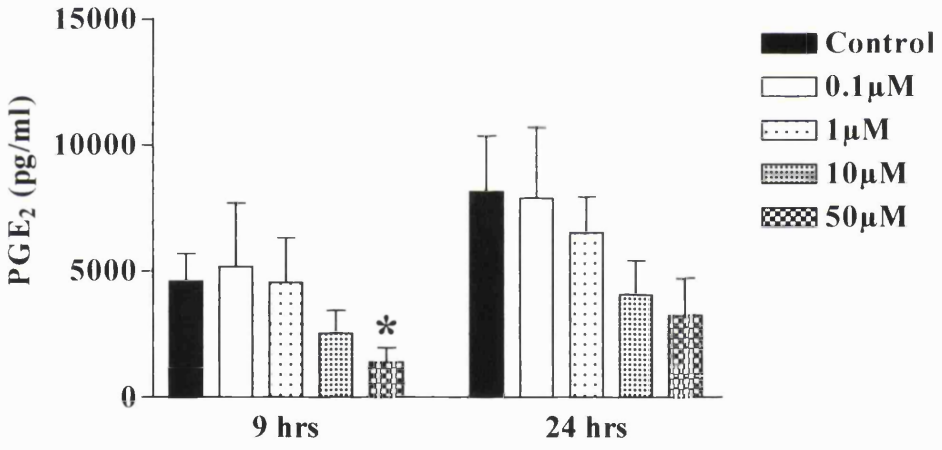


FIGURE 57. Accumulation of PGE_2 in the culture medium of eNOS WT bone marrow-derived macrophages activated with LPS (100ng/ml) in the absence and presence of increasing concentrations of forskolin (0.1-50μM). PGE_2 was measured by ELISA. Data are represented as the mean ± SEM PGE_2 production under each condition (n = 3); * $p < 0.05$ vs control.

EFFECT OF THE ADENYLATE CYCLASE ACTIVATOR, FORSKOLIN, ON COX-1 EXPRESSION IN BONE-MARROW DERIVED MACROPHAGES

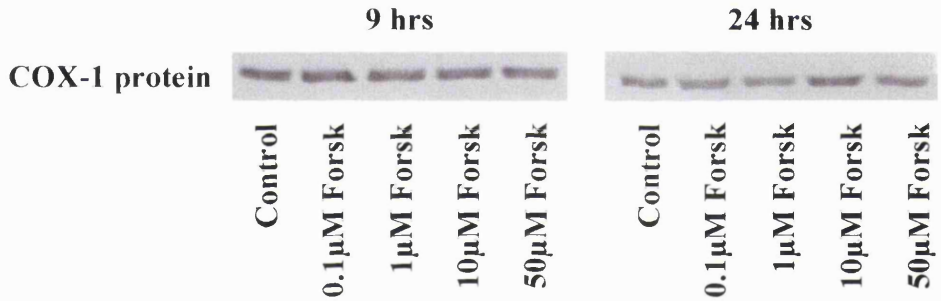


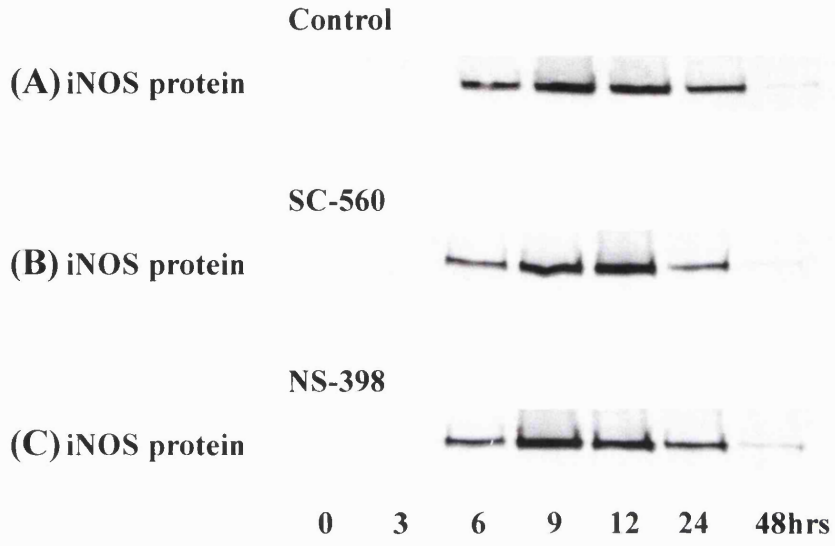
FIGURE 58. Expression of COX-1 protein in eNOS WT bone marrow-derived macrophages activated with LPS (100ng/ml) in the absence and presence of increasing concentrations of forskolin (0.1-50μM). Protein expression was analysed by Western blot. Data are representative of three separate experiments.

3.11 EFFECT OF SELECTIVE COX INHIBITORS ON iNOS AND COX-2 EXPRESSION AND ACTIVITY

To investigate the role of COX isozymes and products on macrophage activation and pro-inflammatory protein expression, RAW 264.7 cells were activated in the presence and absence of the COX-1 selective inhibitor, SC-560 (300nM), or the COX-2 selective inhibitor, NS-398 (30μM), before activation with LPS (1μg/ml). The concentrations selected were known to be effective in the selective inhibition of each isoform (Futaki *et al.,* 1994; Smith *et al.,* 1998). Both iNOS and COX-2 protein expression were unaltered in the presence of these drugs (Figure 59 and 60). The production of nitrite was unaltered by the COX inhibitors (Figure 59).

The measurement of PGE_2 concentration in the presence of either drug indicates that COX-2 is the main source of the prostaglandin as its production is completely abolished by the COX-2 selective inhibitor, NS-398. A large reduction in PGE_2 is also observed when cells are activated in the presence of the COX-1 selective inhibitor, SC-560 but this may be due to non-specific activity of the drug (Figure 60).

EFFECT OF THE COX-1 INHIBITOR, SC-560, AND COX-2 INHIBITOR, NS-398, ON iNOS EXPRESSION AND ACTIVITY IN RAW 264.7 CELLS



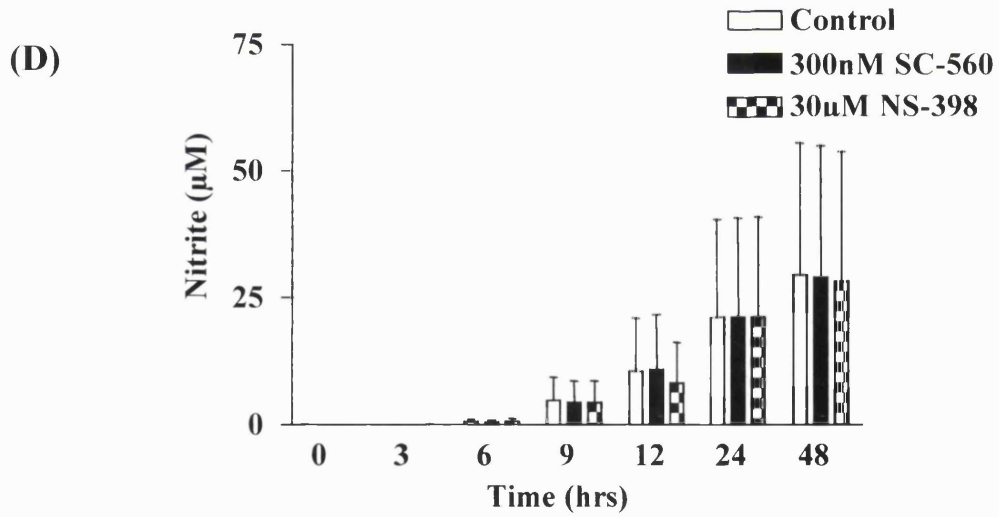
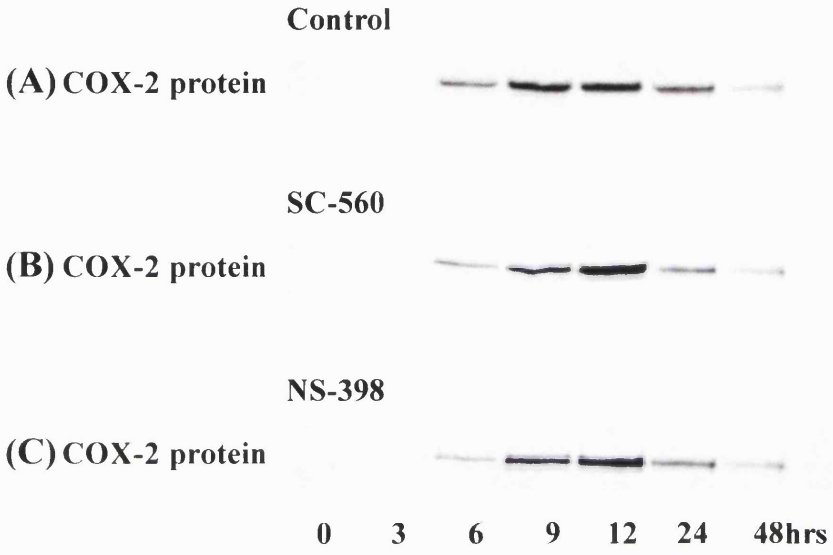


FIGURE 59. iNOS protein expression and activity in RAW 264.7 macrophages activated with LPS (1μg/ml) under control conditions (A) or in the presence of SC-560 (300nM; B) or NS-398 (30μM; C). Protein expression was analysed by Western blot. Accumulation of nitrite in the culture medium was measured by Griess reaction (D). Data are represented as the mean ± SEM nitrite production under each condition (n = 3).

EFFECT OF THE COX-1 INHIBITOR, SC-560, AND COX-2 INHIBITOR, NS-398, ON COX-2 EXPRESSION AND ACTIVITY IN RAW 264.7 CELLS



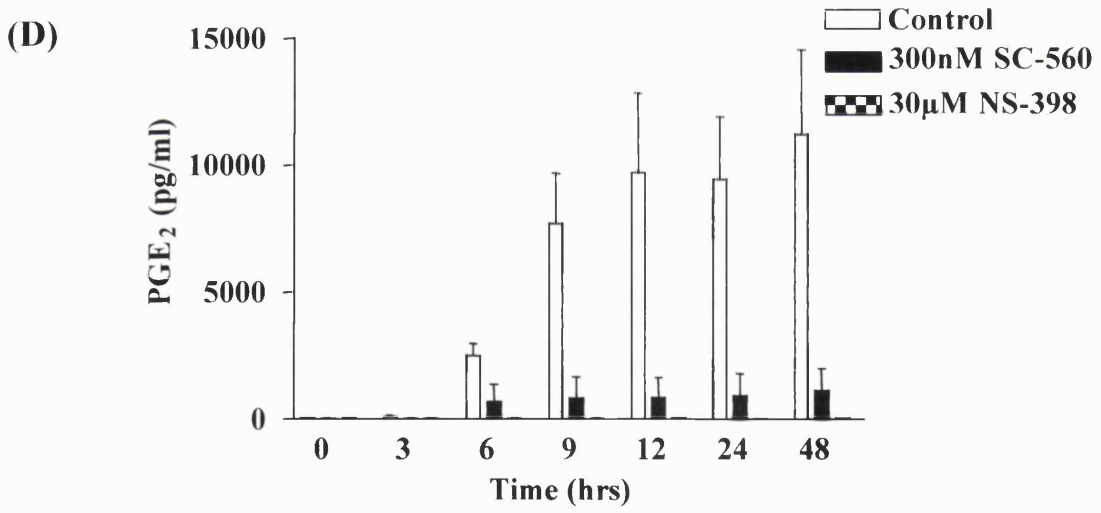


FIGURE 60. COX-2 protein expression and activity in RAW 264.7 macrophages activated with LPS (1μg/ml) under control conditions (A) or in the presence of SC-560 (300nM; B) or NS-398 (30μM; C). Protein expression was analysed by Western blot. Accumulation of PGE_2 in the culture medium was measured by ELISA (D). Data are represented as the mean ± SEM PGE_2 production under each condition (n = 3).

CHAPTER FOUR
DISCUSSION

4. DISCUSSION

4.1 BACKGROUND AND AIM OF CURRENT STUDY

The activation of macrophages with LPS and cytokines induces the expression of iNOS; the product of this enzyme, NO, acts as a cytotoxic and cytostatic effector molecule (MacMicking *et al.*, 1997). Unlike the constitutive isozymes, iNOS is not expressed under basal conditions and the activity of this isoform is independent of increases in intracellular Ca^{2+} (Cho *et al.*, 1992). Thus, activity is regulated predominantly through the control of enzyme expression. The aberrant production of NO by iNOS can lead to local tissue damage and such an effect is thought to contribute to the pathologies of disorders including rheumatoid arthritis, multiple sclerosis and septic shock (McInnes *et al.*, 1996; Titheradge, 1999; Liu *et al.*, 2001). Therefore, the mechanisms responsible for the control of iNOS expression are essential in preventing excessive NO production and an understanding of such control pathways may provide novel targets for drug design.

It is now apparent that during inflammatory episodes NO can feed back to regulate its own production. This can occur via a biochemical mechanism whereby NO inhibits iNOS activity by binding to the haem prosthetic group of the enzyme (Assreuy *et al.*, 1993; Griscavage *et al.*, 1993). Alternatively, NO exerts control over the expression of iNOS (Park *et al.*, 1994; Colasanti *et al.*, 1995; Sheffler *et al.*, 1995; Perez-Sala *et al.*, 2001) and other pro-inflammatory proteins such as COX-2, IL-6 and TNF-α (Salvemini *et al.*, 1993; Deakin *et al.*, 1995; Diaz-Cazorla *et al.*, 1999). One mechanism by which NO may exert the later of these effects is via modulation of the activity of the transcription factor, NF-κB, a pivotal factor in the induction of pro-inflammatory protein expression. Previous reports have hinted that NO may regulate NF-κB activity, but conflicting data have been obtained, in that NO can have both positive (Lander *et al.*, 1993) and negative (Spiecker *et al.*, 1997; DelaTorre *et al.*, 1999) effects. Moreover, studies have suggested that the way in which NO modulates the activity of NF-κB is multi-faceted, with evidence of direct actions of NO in addition to mechanisms involving production of the second messenger cGMP (Matthews *et al.*, 1996; Tetsuka *et al.*, 1996). The investigations described in this

thesis were designed to elucidate more fully the mechanisms by which NO can regulate NF-κB activity and subsequent pro-inflammatory protein expression.

4.2 SUMMARY OF KEY RESULTS

In the present study the existence of a mechanism by which NO both potentiates and inhibits the activity of NF-κB is demonstrated, with lower concentrations of NO stimulating and higher concentrations being inhibitory. As a consequence, NO can both up- and down-regulate pro-inflammatory protein expression. This mechanism enables NO to regulate its own production through effects on iNOS expression. Moreover, via this biphasic effect on NF-κB, NO can turn on and off the expression of other pro-inflammatory proteins, including COX-2 and IL-6, and regulate the activation profile of the macrophage. This feedback loop is easy to reconcile with the needs of host defence to produce a rapid response to a pathogenic stimulus that is shut down promptly so as to minimise (host) tissue damage.

The current study also demonstrates the (patho)physiological significance of a constitutive NOS isoform in the macrophage, which appears crucial in modulating LPS-stimulated expression of iNOS and COX-2. The use of sGC activators and inhibitors indicates a role for sGC and cGMP in the regulation of protein expression by NO. Therefore, NO may exert distinct effects on NF-κB, and subsequent protein expression, by the employment of cGMP-dependent and -independent mechanisms. These findings also provide evidence for an interplay between the NOS and COX enzyme systems, a phenomenon which has previously been investigated with conflicting results (Salvemini *et al.*, 1993; Swierkosz *et al.*, 1995; Hamilton & Warner, 1998). NO is shown to both up- and down-regulate COX-2 expression, as a result of its biphasic effect on NF-κB, and modulate COX-2 activity and PGE_2 production. PGE_2 can lead to the up-regulation of cAMP and through the use of the adenylate cyclase activator, forskolin, a further regulation of LPS-stimulated iNOS and COX-2 expression by cAMP has been established.

4.3 EFFECT OF NO ON NF-κB

In LPS-activated cells, NF-κB has been shown to be the key transcription factor in the regulation of iNOS expression (Xie *et al.*, 1994) and is also one of the factors controlling the expression of COX-2 (Hwang *et al.*, 1997) and IL-6 (Zhang *et al.*, 1994). Therefore, it is possible that NO can exert effects on the expression of these proteins by modulating the activity of NF-κB, a mechanism which has been suggested in previous studies (Colasanti *et al.*, 1995; Park *et al.*, 1997).

In order to test the hypothesis that NO can modulate the expression of iNOS, COX-2 and IL-6 via an effect on NF-κB, macrophages were activated with LPS and endogenous NO production inhibited either concomitantly or 9 hrs subsequently. A lack of NO at the time of LPS administration resulted in a decreased activation of NF-κB. In contrast, inhibition of NOS at later stages led to a prolonged activation of this transcription factor. This demonstrates the biphasic nature of the effect of NO, with early NO production potentiating NF-κB activity, while at later stages of activation NO is necessary to down-regulate NF-κB. This represents a mechanism by which NO can turn on and off the expression of pro-inflammatory proteins, under the control of NF-κB, as part of an endogenous regulation of macrophage activation.

After the biphasic effect of NO on NF-κB and pro-inflammatory protein expression had been established, the way in which NO was able to exert two distinct effects on the same factor was investigated. In order to examine whether this phenomenon was concentration-dependent the effect of different concentrations of exogenous NO on LPS-induced NF-κB activation was investigated. The NO donor, DEA-NO, was selected as it is known to decompose spontaneously in aqueous solution to yield free NO with a half-life of 2-3 mins (Morley & Keefer, 1993). It was found that lower concentrations of DEA-NO (30nM to 3μM) potentiated the activation of NF-κB by LPS while a higher concentration (300μM) was inhibitory. Thus, the different effects of low and high concentrations of NO on NF-κB activity provide an explanation for the ability of NO to exert both positive and negative effects on this transcription factor.

These results agree with previous findings of a biphasic effects of NO on TNF-α stimulated NF-κB activity (Umansky *et al.*, 1998) and also reconcile conflicting reports of both positive and negative effect of NO on the activity of NF-κB (Lander *et al.*, 1993; Peng *et al.*, 1995; Matthews *et al.*, 1996; DelaTorre *et al.*, 1997; DelaTorre *et al.*, 1999; Simpson & Morris, 1999). Several putative mechanisms may explain the inhibitory effects observed at higher concentrations of NO, which might occur at later time points following iNOS expression and 'high output' NO. NO has been shown to inhibit the activation of NF-κB by the stabilisation of IκBα, which occurs via a stimulation of the re-synthesis of this inhibitory protein (Peng *et al.*, 1995; Spiecker *et al.*, 1997). It has also been demonstrated that NO donors, as well as LPS-stimulated NO production, can lead to the S-nitrosation of Cys^{62} on the p50 subunit of NF-κB and this inhibits the DNA binding activity of the transcription factor (Matthews *et al.*, 1996; DelaTorre *et al.*, 1997; DelaTorre *et al.*, 1999). The TNF-α stimulated activation of NF-κB can also be blocked by NO (as released by SNAP) and this effect is reversed by the addition of H_2O_2 (Garban & Bonavida, 2001). Here, NO may be interacting with O_2^- to form $ONOO^-$, thus preventing the conversion of O_2^- to H_2O_2 - a ROI known to stimulate NF-κB activation (Kaul & Forman, 1996).

The potentiating effect of NO at lower concentrations on NF-κB, however, remains to be explained. One possibility is that NO acts by modulating the upstream signalling molecules involved in the pathway of LPS-stimulated NF-κB activation. For example, in TC10 endothelial cells transiently transfected with IKKα, GTN alone has no effect but increases the ability of IKKα to phosphorylate IκBα when administered in combination with TNF-α. This is reflected in a potentiation of TNF-α-stimulated NF-κB activity (Umansky *et al.*, 1998). Therefore NO may act directly on IKK to increase phosphorylation and degradation of IκBα. Low concentrations of NO may also act to stimulate other signalling molecules such as $p21^{ras}$ or MAPK. $p21^{ras}$ is involved in the LPS-induced activation of NF-κB (Pahan *et al.*, 2000) and is also sensitive to modulation by NO, with lower concentrations enhancing the exchange of GDP for GTP and activating the protein (Lander *et al.*, 1995a) through the S-nitrosation of Cys^{118} (Lander *et al.*, 1995b). A more recent study has linked this stimulation to NO produced by constitutive NOS in that NMDA stimulation of

neuronal cultures induces $p21^{ras}$ activation in a manner which is blocked by L-NAME treatment or the use of cultures from $nNOS^{-/-}$ animals (Yun *et al.*, 1998). This may represent an explanation for the results obtained in the current study, with NO produced by a constitutive NOS, possibly eNOS, potentiating the LPS activation of NF-κB via an effect on $p21^{ras}$. This is supported by the findings that the p42/44, p38 and JNK MAPK pathways are stimulated by low concentrations of NO with this effect blocked by farnesyl transferase inhibitors (Lander *et al.*, 1996). Thus, upstream activators such as $p21^{ras}$, which require membrane targeting by farnesylation for activation, are necessary to observe the modulatory actions of NO on MAPKs. These effects of NO are relevant to the current study as MAPKs, such as p38, are known to be involved in induction of iNOS expression in LPS-stimulated RAW 264.7 macrophages (Chen & Wang, 1999). It is also possible that NO may down-regulate such signalling molecules in order to halt cellular activation.

Since many of the molecules that are activated in response to LPS stimulation have only recently been identified, such as MyD88 and IRAK, these also provide targets for future studies to determine the modulation of signalling protein activity by NO.

4.4 EFFECT OF NO ON iNOS

4.4.1 Effect of endogenous and exogenous NO on iNOS expression

The effect of both endogenous and exogenous NO on iNOS expression was investigated in order to demonstrate that the biphasic effect of NO on NF-κB activity was reflected downstream in pro-inflammatory protein expression. Murine macrophages were activated under situations where endogenous NO synthesis was blocked and the effect this had on the profile of iNOS expression was investigated. Under control conditions the presence of LPS elicits an increase in iNOS expression which peaks at between 12 and 24 hrs and then falls again with little or no protein remaining at 96 hrs. The presence of the iNOS-selective inhibitor, 1400W, caused an increase in the peak level of iNOS expression. However, a much more marked effect was observed when macrophages were activated with LPS in medium lacking L-

arginine, a condition which has previously been demonstrated to prevent endogenous NO synthesis by iNOS (Assreuy & Moncada, 1992). When cells were incubated in L-arginine free medium for 24 hrs prior to activation there was no protein expression observed; it is possible that the depletion of L-arginine is affecting the metabolism of the cells such that they are no longer able to synthesise new proteins. When L-arginine free medium was added immediately before LPS activation a shift was observed in the time course and peak expression of iNOS. Initially, expression was delayed, with peak expression occurring 12 to 24 hrs later, after which expression was prolonged as compared to control. Additionally, a reduction was observed in peak protein expression in the absence of L-arginine. A similar effect was also observed in the presence of the NOS inhibitor, L-NAME, although in this situation the shift in expression patterns was not so pronounced, probably due to only partial suppression of endogenous NO synthesis.

These results indicate an important role for NO in the activation of iNOS expression, with NO being required initially to up-regulate, and at later points to rapidly turn off, expression. Furthermore, the differing results observed with the iNOS-selective and non-selective NOS inhibitors suggest that both constitutive and inducible NOS isoforms might have a role in this process.

The biphasic nature of the effect of endogenous NO on iNOS expression was confirmed in an experiment where cells were activated with LPS and NO production blocked concomitantly or after 9 hrs with the NOS inhibitor L-NAME. The inhibition of NOS at the time of LPS administration led to a reduction in iNOS expression while inhibition of NOS at a later time point prolonged the expression of iNOS. These results reflect the effects observed on NF-κB activity under the same conditions confirming that NO exerts its biphasic effect on iNOS expression via a modulation of NF-κB. This link was further strengthened by the observation that the NO donor, DEA-NO, had the same effect on LPS-stimulated iNOS mRNA and protein expression as it did on NF-κB activity, with lower concentrations of DEA-NO augmenting expression while higher concentrations had a negative effect.

These results agree with a previous report of a biphasic effect of NO on iNOS expression (Sheffler *et al.*, 1995) and explain conflicting results which have reported positive and negative effects of NO on the expression of iNOS (Park *et al.*, 1994; Colasanti *et al.*, 1995; Perez-Sala *et al.*, 2001). These contradictory reports may have occurred due to the investigation of only one time point during activation; the current study demonstrates that NO potentiates protein expression at early time points and is inhibitory later, an effect only apparent when expression is followed over a range of time points. Furthermore, only the positive or negative effect may have been observed by the employment of single concentrations of NO. It is clearly important in such studies to be aware of the concentrations of NO released by specific NO donor compounds, since different concentrations of NO have markedly varied modulatory effects.

Low concentrations of NO gas or NO donor have previously been shown to stimulate NF-κB activity and TNF-α production in human peripheral blood mononuclear cells in the absence of another inflammatory stimulus (Lander *et al.*, 1993). NF-κB activation and COX-2 expression has also been demonstrated with the NO donor GSNO in RAW 264.7 cells (von Knethen *et al.*, 1999). In the current study, iNOS expression was assessed after 24 hrs exposure to NO alone and this was ineffective in inducing pro-inflammatory protein expression. However, this may have been too early for expression to be detected. In the absence of co-stimulation with LPS, NO may have a weak effect and protein expression might only be observed at a later time point. Alternatively, the concentrations of NO used in the current study may have been insufficient to initiate activation, although the concentration of NO released (2μM) in this study by the highest concentration of DEA-NO employed is equivalent to pathological concentrations (Wink & Mitchell, 1998).

4.4.2 Role of constitutive NOS in iNOS expression

In the current study, the peak concentrations of NO released by different concentrations of DEA-NO were measured using a specific NO electrode and ranged between 1nM and 2μM. The NO release from the concentrations of DEA-NO which had a potentiating effect on NF-κB activity and iNOS expression correspond to the

approximate physiological concentrations of NO released by a constitutive NOS isoform (nM range); the inhibitory effects of DEA-NO occurred at a concentration that might be observed under pathological conditions as derived from iNOS (≥1μM; Wink & Mitchell, 1998). Such findings indicated the possibility of a constitutive NOS involved as a source of low concentrations of NO.

Previous reports in other cells types indicate that iNOS expression and NF- κB activity can be modulated by constitutive NOS, in particular nNOS. In glial cells it has been reported that NO produced by nNOS acts to suppress NF-κB activity and iNOS expression under resting conditions (Colasanti *et al.*, 1997; Togashi *et al.*, 1997). It has also been shown that stimulation with LPS and IFN-γ inhibits basal NOS activity in these cells by a tyrosine phosphorylation-dependent mechanism and this is followed by the expression of iNOS mRNA (Colasanti *et al.*, 1999). A constitutive eNOS-type protein has been demonstrated in macrophages and monocytes (Hecker *et al.*, 1992; Reiling *et al.*, 1994; Roman *et al.*, 1997; Miles *et al.*, 1998) yet previous reports have not identified a role for this enzyme. It was apparent from the concentrations of NO which potentiated NF-κB activity, as well as the differing effects of iNOS-selective and non-selective NOS inhibitors on the expression profile of pro-inflammatory protein expression, that a constitutive NOS isoform may be important in the biphasic effect of NO. Additionally, effects of L-NAME on NF-κB activity were observed after 90 mins confirming the importance of early NO production at a time point when iNOS expression/activity cannot be detected. Expression of iNOS is not observed by Western blot under basal conditions, and the concentrations of LPS in the culture medium are 100-fold lower than the minimal LPS concentrations with which iNOS expression was observed. Also, LPS-stimulated expression of iNOS was not observed until 3-6 hrs after stimulation. These findings reiterated the possibility that a constitutive NOS isoform may be involved in macrophage activation.

In Western blots of extracts from murine macrophages it was shown that the cells expressed eNOS but not nNOS, suggesting the former may be the more important isozyme. In order to determine a role for eNOS in macrophage activation, murine macrophages were transfected with eNOS to investigate the effect that increased

levels of this protein would have on LPS-induced iNOS expression. Although Western blots clearly indicate the successful transfection of eNOS, the effects on iNOS expression were inconsistent.

In an alternative approach, bone marrow-derived macrophages were isolated from eNOS WT and KO mice and differences in LPS-stimulated iNOS expression were investigated. These cells were characterised by flow cytometry to demonstrate that the culture method led to the development of monocytic cells, and immunoblotting was used to ensure that there was no expression of the deleted gene. The expression of eNOS in WT, but not KO, cells was demonstrated by immunoblot, confirming the presence of eNOS in macrophages. Activation with LPS revealed a distinct difference in the expression of iNOS between WT and KO cells. In eNOS KO cells a reduced level of iNOS expression and activity was observed over 48 hrs. Therefore, NO production from eNOS augments the expression and activity of iNOS. This observation is in accord with the hypothesis that the potentiating effect of NO on NF-κB and pro-inflammatory protein expression might be eNOS-derived. The effect on iNOS activity was less pronounced than that observed on iNOS expression. The reasons for this are not clear but may reflect a balance that is reached between a diminished NO output from the lost protein and a slight reduction in the inhibitory effect of NO on NOS activity as a result of lower iNOS expression.

Although a potential role for eNOS in macrophage activation has been established, the mechanism(s) by which eNOS is activated remains to be investigated. It has previously been reported that RAW 264.7 macrophages, as used in the current study, produce NO under basal conditions and that this NO production is Ca^{2+}-dependent (Schmidt *et al.*, 1992). This would suggest that in these macrophages constitutive NOS is active in the absence of stimulation. Alternatively, LPS may stimulate eNOS activity via increasing the concentrations of intracellular Ca^{2+}. The Ca^{2+} channel antagonists diltiazem, nifedipine and verapamil increase cytoplasmic levels of IκBα and reduce nuclear levels of NF-κB p65 subunit and subsequent iNOS expression in LPS-treated rat Kupffer cells (Mustafa & Olson, 1999). This suggests that an increase in Ca^{2+} levels is involved in LPS-induced NF-κB activation and expression of iNOS. In a recent study using the J774 murine macrophage cell line it was found that the

Ca^{2+}-ATPase inhibitor, thapsigargin, and the Ca^{2+} ionophore, A23187, increase concentrations of nitrite produced by low levels of LPS but inhibit nitrite production when co-administered with high doses of LPS (Korhonen *et al.*, 2001). The potentiating effect of these compounds is only apparent when they are added an hour before or after LPS treatment while the inhibitory effect still occurs with addition at later time points. Such observations support a role for Ca^{2+}-dependent eNOS activation in augmenting the LPS response.

Although the addition of compounds which modulate intracellular Ca^{2+} levels can affect iNOS expression and activity, it has also been reported that LPS itself does not trigger changes in intracellular Ca^{2+} levels, suggesting alternative mechanisms for eNOS activation may be important (Raddassi *et al.*, 1994; Korhonen *et al.*, 2001). eNOS can also be activated by phosphorylation via the PI-3K/Akt pathway in response to shear stress in endothelial cells (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999) and this pathway may also exist in macrophages. LPS increases the activity of PI-3K in RAW 264.7 murine macrophages although inhibition of PI-3K leads to an increase in iNOS protein expression and activity (Diaz-Guerra *et al.*, 1999). In contrast, a recent study has shown that vascular endothelial growth factor (VEGF) stimulates ICAM-1 expression via a PI-3K/Akt/NO-dependent process in brain microvascular endothelial cells (Radisavljevic *et al.*, 2000). As ICAM-1 is up-regulated by NF-κB, this represents evidence that NO from phosphorylation-activated eNOS might activate this transcription factor - a pathway that may be utilised by LPS.

Although LPS may affect eNOS activity it may also regulate the expression of this enzyme. For example, LPS-treatment of rats leads to an increase in brain eNOS mRNA levels with a corresponding rise in Ca^{2+}-dependent NOS activity (Iwase *et al.*, 2000). Furthermore, while iNOS requires higher levels of LPS to induce expression, augmentation of eNOS mRNA expression is obtained with low doses of LPS, suggesting that up-regulation of constitutive NOS may be a preliminary event in inflammation. In bovine coronary venular endothelial cells, LPS treatment enhances the activation of eNOS observed with bradykinin or Ca^{2+} ionophore over a 10 hour period, although after 10 hours of treatment LPS leads to a decrease in levels of eNOS protein (Huang *et al.*, 1998). In the current study, the treatment of cells with LPS did

not lead to any difference in levels of eNOS expression. This may indicate that such a mechanism is not relevant to the role of eNOS in macrophages or it is possible that an up- or down-regulation is only apparent before or after the 24 hr time point studied.

4.4.3 Effect of cGMP on iNOS expression and activity

One of the main pathways initiated by NO is the activation of sGC and the production of the second messenger cGMP, which modulates cellular function via proteins such as G-kinase (Hobbs & Ignarro, 1996). It has recently been demonstrated that the sGC enzyme in intact cells is activated by NO with an $EC_{50} \leq 20nM$ (Bellamy *et al.*, 2000), which corresponds with the concentrations of NO which were shown to potentiate LPS-induced NF-κB activity and pro-inflammatory protein expression in this study. Therefore, the sGC activator BAY 41-2272 and sGC-selective inhibitor ODQ were used in eNOS WT bone marrow-derived macrophages to investigate whether cGMP was involved in LPS-stimulated iNOS expression. An increase in iNOS expression and activity was observed in the presence of BAY 41-2272 while a decrease occurred in the presence of ODQ. This suggests that cGMP is involved, at least in part, in LPS-stimulated iNOS expression. These findings are in agreement with previous reports that cGMP has a positive role in iNOS expression (Perez-Sala *et al.*, 2001). In the present study, an activator of sGC potentiated LPS-induced iNOS expression, while an sGC-specific inhibitor slightly decreased iNOS expression. Consequently, it is possible that the potentiating effects of NO on NF-κB activity and subsequent protein expression described in this thesis occur, at least in part, via activation of sGC and the production of cGMP. Interestingly, a negative effect of cGMP on LPS-stimulated NF-κB activity is observed with ANP, which acts on the particulate guanylate cyclase (Kiemer & Vollmar, 1998); this may be due to the presence of different pools of cGMP.

The current findings are supported by evidence that macrophages produce cGMP in response to NO. In RAW 264.7 cells, basal NO production is accompanied by the release of small amounts of cGMP, although further increases in cGMP are not apparent after IFN-γ and LPS treatment (Schmidt *et al.*, 1992). Therefore, basal concentrations of NO may initially potentiate the activation of the macrophage

through increases in cGMP levels but the constitutive NOS may then be turned off by the LPS or cytokine treatment.

There are a number of means by which cGMP may potentially exert its effects, including activation of G-kinase, cyclic nucleotide gated ion channels and modulation of PDEs (Hobbs & Ignarro, 1996; Hobbs, 1997). In primary glial cell cultures cGMP analogues and the PDE 5 inhibitor, zaprinast, increase LPS-stimulated NO release and iNOS expression while the G-kinase inhibitor, KT5823 has an inhibitory effect (Choi *et al.*, 1999). This indicates that cGMP is augmenting LPS-induced iNOS expression through activation of G-kinase. Moreover, the NO donor SNAP activates NF-κB and induces TNF-α mRNA and protein expression in feline cardiac myocytes, and this is blocked by pre-treatment with ODQ or a G-kinase inhibitor (Kalra *et al.*, 2000). Furthermore, G-kinase has also been shown to phosphorylate an IκBα-GST fusion protein on Ser^{32} *in vitro* (Kalra *et al.*, 2000). As Ser^{32} is one of the residues which is phosphorylated before proteasomal degradation of IκBα (Traenckner *et al.*, 1995), this represents a mechanism by which the sGC-cGMP pathway could initiate the activation of NF-κB, via a G-kinase dependent process. LPS-stimulation of rat cerebellar astrocyte-enriched primary cultures leads to an increase in cGMP levels which peaks at between 9 and 12 hrs and then falls sharply by 48 hrs. Concomitantly, LPS causes a gradual decrease in the expression of the sGC β_1 subunit, with levels of this protein decreasing by 60-70% after 20 hrs of LPS stimulation (Baltrons & Garcia, 1999). Therefore, it seems that while LPS can stimulate cGMP production this occurs at earlier time points of activation and at later stages the sGC enzyme is turned off by the down-regulation of protein expression. This represents a way by which the potentiating effects of NO on pro-inflammatory protein expression, by the activation of sGC and production of cGMP, are blocked at later stages when pro-inflammatory proteins must be down-regulated.

NO may exert different effects on the same protein by cGMP-dependent and -independent mechanisms. In rat mesangial cells GSNO leads to both a rapid and delayed phase of p42/44 MAPK activation (Callsen *et al.*, 1998). The early activation of MAPK after 5 mins is also apparent with 8-bromo-cGMP and the effects of GSNO at this time can be blocked with the sGC inhibitor, NS 2028. In contrast, the delayed

phase of MAPK activation is unaffected by NS 2028 (Callsen *et al.*, 1998). This suggests that NO regulates MAPKs by both cGMP-dependent and independent mechanisms. The role that such a mechanism may play in the biphasic regulation of NF-κB remains to be established.

4.5 EFFECT OF NO ON IL-6

IL-6 is another pro-inflammatory protein which is up-regulated in response to LPS by the activation of NF-κB (Zhang *et al.*, 1994). In order to demonstrate that the biphasic effect of NO on NF-κB was apparent in other proteins controlled by this transcription factor, the expression of IL-6 was followed in cells activated under conditions where endogenous NO production was inhibited. Under these conditions a shift was observed in IL-6 production in that initially there was a reduction in IL-6 levels but at later time points an increased level of IL-6 production was observed in the absence of NO. Therefore, early NO production augments IL-6 expression while at later stages NO is required to down-regulate IL-6 production. Thus, the biphasic effect of NO on NF-κB activity is mirrored in IL-6 production suggesting that this pro-inflammatory protein is also subject to NO-mediated regulation in an identical fashion to iNOS.

4.6 EFFECT OF NO ON COX-2

4.6.1 Effect of endogenous and exogenous NO on COX-2 expression and activity

The expression of COX-2, which is also modulated by NF-κB (Hwang *et al.*, 1997), was investigated under situations where endogenous NO synthesis was prevented. Under control conditions the presence of LPS elicits an increase in COX-2 expression which peaks at between 12 and 24 hrs and then falls again with little or no protein remaining at 96 hrs. The presence of the iNOS-selective inhibitor, 1400W, caused a decrease in peak levels of COX-2. Moreover, as is the case for iNOS, when cells were activated with LPS in L-arginine free medium a shift was observed in both the time course and peak expression of COX-2. Initially, expression was delayed with peak expression occurring 12 to 24 hrs later after which expression was prolonged

compared to control. Additionally, a reduction was observed in peak protein expression in the absence of L-arginine. A similar effect was also observed in the presence of the NOS inhibitor, L-NAME, although, as with iNOS, the shift in expression patterns was not so pronounced. The administration of LPS in the presence of increasing concentrations of DEA-NO resulted in a similar effect on COX-2 expression as was observed with NF-κB activity and iNOS expression. At low concentrations DEA-NO augmented COX-2 mRNA and protein expression while at higher concentrations expression was inhibited. These results demonstrate that the biphasic effect of NO on NF-κB is reflected in COX-2 expression.

As with iNOS and NF-κB these findings explain previous experiments which have reported that NO can both positively and negatively regulate COX-2 expression (Tetsuka *et al.*, 1996; Patel *et al.*, 1999) and are in agreement with a study reporting a biphasic effect of NO on COX-2 (Diaz-Cazorla *et al.*, 1999).

NO also appears important for the activity of COX-2 with levels of PGE_2 failing to reach those of control when endogenous NO production was prevented by the removal of L-arginine. Previous studies which have investigated COX-2 activity in iNOS KO cells agree with the current findings that NO from iNOS enhances COX-2 activity (Nogawa *et al.*, 1998; Marnett *et al.*, 2000). NO has also been shown to stimulate COX activity in RAW 264.7 cells (Salvemini *et al.*, 1993). A number of different mechanisms have been suggested to explain the effect of NO on COX activity. The effect of NO on COX activity may be dependent on the presence of a particular NO-derived species. Peroxynitrite, which is formed from the reaction between NO and O_2^-, has been found to modulate COX activity. Indeed, in RAW 264.7 cells activated with LPS and IFN-γ, agents that lower levels of O_2^- caused a dose-dependent inhibition of prostaglandin formation while COX-2 expression remained unaltered (Landino *et al.*, 1996). Additionally 3-morpholinosydnonimine (SIN-1), which decomposes to release NO and O_2^-, was found to stimulate prostaglandin formation from purified COX enzymes and from COX-1 in rat aortic smooth muscle cells (Landino *et al.*, 1996; Upmacis *et al.*, 1999). Furthermore, a study investigating the effect of different NO donor compounds on PGE_2 release from

LPS-treated astrocytes showed that donors which released $NO^{\cdot}$ or NO^{+} enhance PGE_2 production (Vidwans *et al.*, 2001).

4.6.2 Effect of constitutive and inducible NOS on COX-2 expression and activity

Since constitutive NOS was shown to modulate the expression and activity of iNOS, bone marrow-derived macrophages were isolated from eNOS WT and KO mice and differences in LPS-stimulated COX-2 expression were investigated. In eNOS KO cells an increased level of COX-2 expression and activity was apparent compared with WT. Therefore, while NO production from eNOS augments the expression of iNOS, it inhibits the expression of COX-2. These experiments were repeated with macrophages derived from the bone marrow of iNOS WT and KO animals in order to investigate the influence of this isoform on COX-2 expression. In iNOS KO cells a slight reduction in LPS-induced COX-2 expression and activity was apparent. Therefore, from these studies, it appears that low concentrations of NO from eNOS inhibit LPS-stimulated COX-2 expression while high concentrations of NO produced by iNOS potentiate COX-2. Although the present study demonstrated that exogenous NO alters COX-2 expression in a similar manner to that of iNOS (presumably via altering NF-κB activity), the deletion of eNOS had contrasting effects to those that might be predicted, suggesting that modulation of NF-κB is not the principal mechanism. NF-κB has been shown to be the main factor in the control of LPS-stimulated iNOS expression (Xie *et al.*, 1994), but for the COX-2 gene NF-IL6 has also been shown to be a key factor in activation by LPS (Wadleigh *et al.*, 2000). In a recent study NO has been shown to regulate COX-2 expression through an effect on both NF-κB and NF-IL6 DNA binding activity (D'Acquisto *et al.*, 2001). This may explain, at least in part, the ability of NO to exert different effects on iNOS and COX-2 expression. It is possible that exogenous NO exerts a biphasic effect on COX-2 expression via a mechanism that is distinct to the alteration brought about by eNOS/iNOS deletion.

4.6.3 Effect of cGMP on COX-2 expression and activity

The effect of BAY 41-2272 on COX-2 expression was also investigated. BAY 41-2272 potentiated COX-2 expression but inhibited PGE_2 production. In a previous study, SNAP potentiation of COX-2 mRNA expression in IL-1β-activated rat primary mesangial cells is blocked by methylene blue (an inhibitor of sGC), and 8-bromo-cGMP (a cGMP analogue) increases COX-2 mRNA levels (Tetsuka *et al.*, 1996). Thus, it appears that while cGMP increases COX-2 expression, the activity of the enzyme is decreased. These contrasting actions may be reconciled by differing effects of NO and cGMP on COX-2 expression and activity. Since cGMP appears to augment iNOS expression, the increased production of NO may then exert independent effects on COX-2 expression and activity. These observations, in tandem with the different effects observed with the eNOS and iNOS KO cells, suggest a much more complex regulatory mechanism for NO on COX-2 expression and activity than that exerted over iNOS.

4.7 EFFECT OF NO AND cGMP ON COX-1 EXPRESSION

In iNOS and eNOS KO cells there was no difference in COX-1 expression, as compared with WT, despite alterations in COX-2 expression. Therefore, it seems that there is no up- or down regulation of this enzyme to compensate for changes in its inducible counterpart. Moreover, BAY 41-2272 did not affect COX-1 expression, suggesting this isoform did not have a significant role to play in PGE_2 generation.

4.8 EFFECT OF cAMP ON iNOS AND COX EXPRESSION AND ACTIVITY

Since PGE_2 production can result in cAMP synthesis, the actions of cAMP on iNOS and COX-2 expression were also investigated to determine if COX-2 activity also regulated the iNOS pathway. After 9 hrs, the adenylate cyclase activator, forskolin, had little effect on LPS-stimulated iNOS expression whereas at 24 hrs forskolin had a concentration-dependent inhibitory effect on iNOS expression and activity. These

results are supported by previous findings indicating that cAMP inhibits iNOS expression (Pang & Hoult, 1997; Mustafa & Olson, 1998). The mechanism by which cAMP alters protein expression remains to be determined. cAMP activates A-kinase and H89, an inhibitor of A-kinase, has been shown to increase LPS-stimulated iNOS mRNA and protein expression in rat astrocytes (Pahan *et al.*, 1997). In the same study, H89 was also reported to increase the expression of iNOS stimulated by TNF-α, IL-1 and IFN-γ, indicating that A-kinase is modulating the activity of a signalling molecule common to each of these pathways. In rat Kupffer cells, forskolin prevents the translocation of the NF-κB p65 subunit and induces the synthesis of IκBα mRNA (Mustafa & Olson, 1998). cAMP may also have post-transcriptional effects as forskolin has been reported to decrease the stability of iNOS mRNA (Mustafa & Olson, 1998). Both of these mechanisms would lead to a decrease in iNOS expression as observed in the current study. Whether any of the above mechanisms are involved in the effects of forskolin on LPS-induced macrophage activation observed in the current study remains to be elucidated.

The lowest concentration of forskolin (0.1μM) augmented LPS-stimulated COX-2 expression after 9 hrs while at higher concentrations (1-50μM), and at 24 hrs, forskolin inhibited the expression and activity of this enzyme. As PGE_2 produced by COX can lead to an up-regulation of cAMP through binding to EP receptors this may represent a self-regulatory mechanism governing COX-2 expression and activity. As the current findings show a potentiation of COX-2 expression by forskolin initially, it is possible that a self-amplifying mechanism occurs at the onset of macrophage activation while at later stages the feedback is down regulatory, akin to NO and iNOS. It has previously been shown that PGE_2 stimulated COX-2 expression in LPS-treated RAW 264.7 cells and this was dependent on stimulation of the EP_2 or EP_4 receptor subtypes and the activation of adenylate cyclase (Hinz *et al.*, 2000b). Similar results have been obtained in studies using murine epidermal keratinocytes (Maldve *et al.*, 2000). Forskolin did not affect the expression of COX-1 at any time point indicating that this gene is not transiently regulated in response to cAMP.

4.9 EFFECT OF COX INHIBITORS ON iNOS AND COX-2 EXPRESSION AND ACTIVITY

In order to examine directly whether the products of the COX enzymes were involved in regulating their own expression, and if a reciprocal regulation existed to control iNOS expression, the effects of selective COX-1 and COX-2 inhibitors were investigated. It was found that these compounds had very little effect on LPS-induced iNOS or COX-2 expression or nitrite production despite concentrations shown to be effective in previous reports (Futaki *et al.*, 1994; Smith *et al.*, 1998) and which significantly reduced PGE_2. This would suggest that the products of the COX enzymes had no effect on transcription factor activation, protein stability or NOS activity. Other studies have reported a lack of effect of COX inhibitors on NOS in agreement with the current findings (Salvemini *et al.*, 1993; Hamilton & Warner, 1998). This is in contrast with reports suggesting that PGE_2 inhibits iNOS expression and NF-κB activity in J774.1 murine macrophages (Pang & Hoult, 1997; D'Acquisto *et al.*, 1998). It is unclear why present results showed no effect. In future studies the use of cells derived from COX-1 and COX-2 KO mice would provide more specific inhibition.

4.10 MODULATION OF OTHER TRANSCRIPTION FACTORS BY NO

Although an effect of NO has been demonstrated on the transcription factor NF-κB, it is possible that NO may also regulate pro-inflammatory protein expression by modulating other transcription factors such as AP-1. Thus, similar experiments could be performed to investigate the effect of NO on such factors. AP-1 comprises homo- and hetero-dimers of the *fos* and *jun* DNA-binding proteins and has a binding site on the iNOS and COX-2 genes (see Chapters 1.2.4 and 1.7.1). This factor also appears to be sensitive to modulation by NO. IFN-γ treatment of primary cultures of human brain-derived cells decreases levels of AP-1 binding to DNA and this effect is blocked by treatment with NOS inhibitors (Conant *et al.*, 1998). It appears that NO may inhibit the DNA-binding activity of AP-1 by interacting with thiol groups, as the inhibitory effect of NO was reversed by DTT treatment or the use of *fos* and *jun* subunits with Cys→Ser mutations (Nikitovic *et al.*, 1998). This may represent a

mechanism distinct from that which has led to reports that the DNA binding activity of AP-1 can be up-regulated by treatment with the NO donor GSNO in RAW 264.7 murine macrophages (von Knethen *et al.*, 1999).

4.11 POST-TRANSCRIPTIONAL MODULATION BY NO

NO may also exert positive and negative effects on pro-inflammatory protein expression by mechanisms which occur at a post-transcriptional level. In human glomerular mesangial cells treated with IL-1β and TNF-α, the presence of L-NAME increases the stability of iNOS mRNA (Perez-Sala *et al.*, 2001). This effect is also apparent in the presence of ODQ, while iNOS mRNA stability is decreased by 8-bromo-cGMP, suggesting a cGMP-dependent down-regulation of iNOS expression at a post-transcriptional level. Whether such an effect is relevant to the present study remains to be determined.

4.12 SIMILAR FINDINGS IN DIFFERENT SYSTEMS

In the current study, through a biphasic effect on NF-κB activity, NO can both up- and down-regulate pro-inflammatory protein expression and consequently macrophage activation. It appears that NO also exerts this biphasic, concentration-dependent effect on related systems. In $CD4^+$ T-cells, SNAP alone has no effect on differentiation into Th_1 cells, but low concentrations of NO enhance interleukin-12 (IL-12)-induced differentiation and IFN-γ production while higher concentrations are inhibitory (Niedbala *et al.*, 1999). This effect is only apparent on the induction of the Th_1 cell subtype and does not occur in established cells. The pathway by which NO exerts this effect has yet to be resolved, but results obtained in the current study may explain, at least in part, the mechanism of action of NO. Activated macrophages produce IL-12, which is responsible for inducing Th_1 cell differentiation. The promoter of the p40 subunit of IL-12 contains an NF-κB site which is essential for up-regulation (Murphy *et al.*, 1995). NO may therefore be instrumental in modulating IL-12 production by macrophages; indeed a potentiating effect of NO on IL-12

production has previously been reported (Rothe *et al.*, 1996). This represents the involvement of NO in a complex feedback loop, as NO may modulate IL-12 production and subsequent IFN-γ production by Th_1 cells. IFN-γ in turn controls induction of iNOS expression and NO synthesis in the macrophage (Weisz *et al.*, 1994). Consequently, NO is involved in the turning on and off of its own production and also exerts a control on inflammation that extends further than the macrophage.

A similar system to that described in the current study may also control IL-1-stimulated bone resorption by osteoclasts, which are bone re-modelling cells that originate from precursor cells of the monocyte/macrophage lineage. Both rat and human osteoclasts express eNOS, and expression of iNOS can be stimulated by cytokine treatment (Brandi *et al.*, 1995). As in the macrophages of the current study it appears that both isoforms exert a control over osteoclast activity, with the non-specific NOS inhibitor L-NMA inhibiting osteoclast resorption of bone, while after 18 hrs of LPS treatment the iNOS-specific inhibitor aminoguanidine enhances osteoclastic bone resorption (Brandi *et al.*, 1995). Therefore, low concentrations of NO, from eNOS, may potentiate bone resorption while higher concentrations of NO, from iNOS, may inhibit osteoclast formation and activation. Further evidence for a control of bone remodelling by NO from constitutive and inducible NOS isoforms is provided by reports of defects in bone remodelling in cells derived from $eNOS^{-/-}$ and $iNOS^{-/-}$ mice (van't Hof *et al.*, 2000; Aguirre *et al.*, 2001; Armour *et al.*, 2001).

4.13 CONCLUSIONS

NO exerts a biphasic effect on NF-κB expression and subsequent expression of pro-inflammatory proteins, with low concentrations of NO potentiating LPS-induced NF-κB activation and protein expression while higher concentrations of NO are inhibitory. In the present study a dual effect of NO is demonstrated and the link between the biphasic modulation of NF-κB activity by NO, and its reflection in the expression of the pro-inflammatory proteins iNOS, COX-2 and IL-6, is established. Indeed these findings indicate that NO, through its effect on NF-κB activity, may be a key regulator of the activation profile of the macrophage.

It has been demonstrated that macrophages express the eNOS isoform and that this, in addition to iNOS, is involved in the modulation of LPS-stimulated iNOS and COX-2 expression. The effect of NO on protein expression occurs, at least in part, through a cGMP-dependent mechanism.

These findings indicate that NO feeds back to regulate the expression of its own synthetic enzyme, initially augmenting its own production in response to inflammatory stimuli then at later stages turning off its own production, possibly to prevent damage to host tissue. An understanding of the mechanism by which NO production is regulated under pathological conditions is crucial to the development of therapies for diseases associated with the aberrant production of NO.

4.14 FUTURE STUDIES

There are a number of potential future studies that arise from the current findings:

1. It remains to be determined whether NO production from eNOS in macrophages is the result of basal activity or stimulation by LPS. A role for Ca^{2+}-dependent or -independent activation of eNOS could be elucidated by the activation of cells with LPS in the presence of Ca^{2+} chelators, calmodulin antagonists or inhibitors of PI-3K.

2. Further investigation is required to demonstrate if these cells express the sGC enzyme and whether the activation of macrophages with LPS leads to an increase in intracellular cGMP levels. Once this has been established, future work also involves elucidating the mechanism by which cGMP can modulate the pathway to iNOS expression.

3. The existence of the modulation of signalling molecules such as $p21^{ras}$ and IKK by NO in the present system remains to be established.

4. The different effects of NO observed on iNOS and COX-2 expression may be due to an effect of NO on other transcription factors. This may be of relevance to the

different mechanisms by which NO controls COX-2 expression. NO may also exert additional post-transcriptional effects that remain to be addressed.

5. In the current study a reduction in PGE_2 production was observed in the absence of endogenous NO but the exact mechanism or NO product responsible for this effect remain to be determined.

6. An effect of cAMP on both iNOS and COX-2 expression and activity was apparent in the results described here. The pathway by which these effects occur requires further study.

7. As the use of inhibitors of both COX isoforms had little effect on iNOS and COX-2 expression, the bone marrow-derived macrophages from COX-1 and COX-2 KO mice would provide a more selective inhibition of each isoform for future experiments.

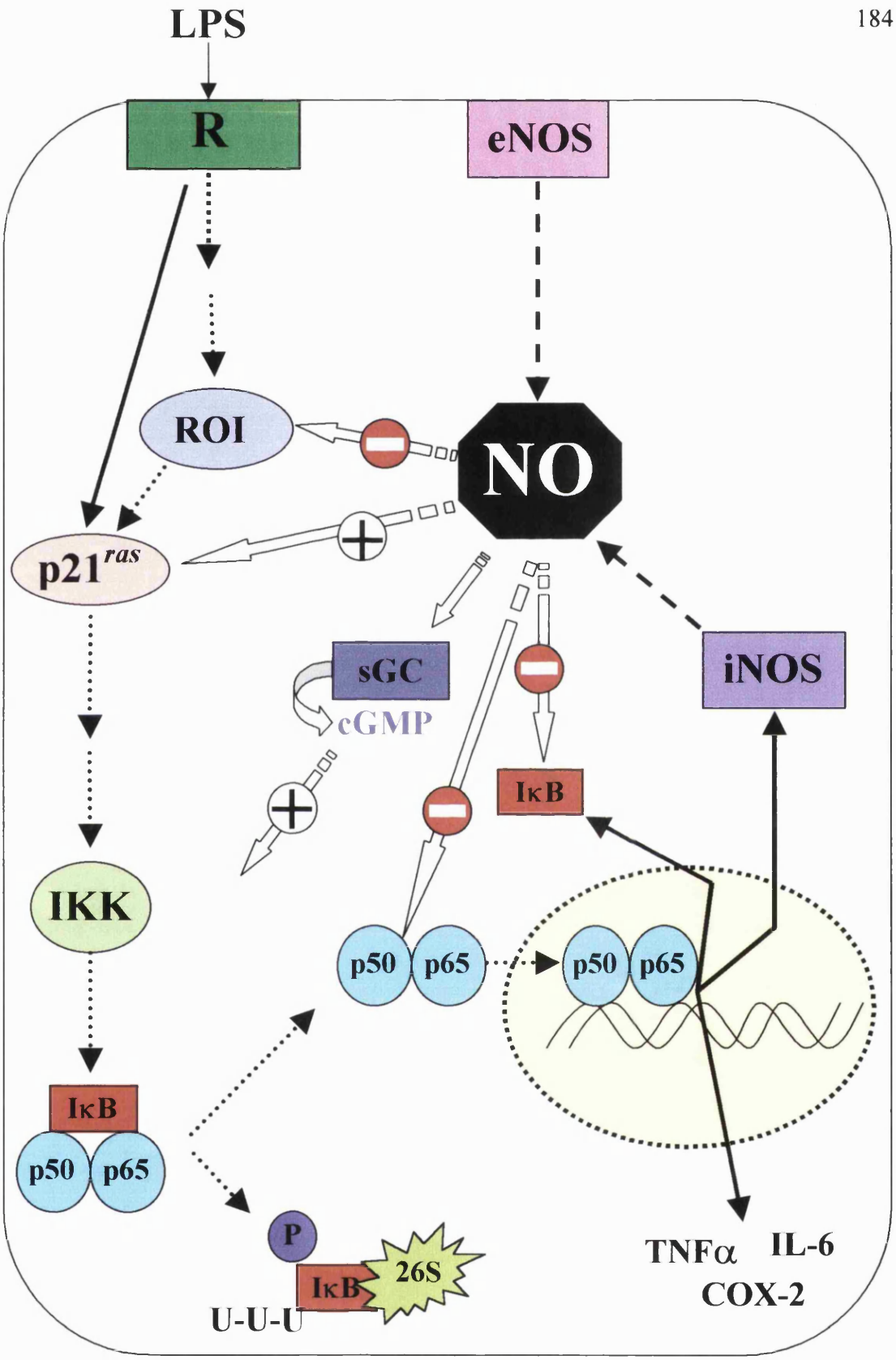


FIGURE 61. Schematic of the possible mechanisms by which NO may exert positive (+) and negative (-) effects on LPS-stimulated NF-κB activation. LPS –lipopolysaccharide; R – receptor; ROI – reactive oxygen intermediates; IKK – IκB kinase; IκB – inhibitor of κB; p50 – NFκB p50 subunit; p65 - NFκB p50 subunit; P – phosphorylation; U – ubiquitin; 26S – 26S proteasome complex; TNFα - tumour necrosis factor alpha; IL-6 – interleukin 6; COX-2 – cyclooxygenase 2; iNOS – inducible nitric oxide synthase; sGC – soluble guanylate cyclase; cGMP – guanosine-3'5'-monophosphate; NO – nitric oxide; eNOS – endothelial nitric oxide synthase

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